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ULSAN NATIONAL INSTITUTE OF SCIENCE AND TECHNOLOGY

Master's Thesis

The effect of Osmolality on the predatory abilities of *Bdellovibrio bacteriovorus*

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Department of Biological Sciences

Graduate School of UNIST

2018

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A thesis/dissertation
submitted to the Graduate School of UNIST
in partial fulfillment of the
requirements for the degree of
Master of Science

Sangmo Son

Jan/3rd/2018

Approved by

Advisor
Robert J. Mitchell

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Sangmo Son

This certifies that the thesis/dissertation of Sangmo Son is approved.

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Abstract

Bdellovibrio-and-Like-Organisms known as BALOs are predatory bacteria. They grow up by predating other Gram-negative bacteria. When they meet prey, they attach to the prey and invade into periplasm. In the periplasm of their prey, they attack the inner membrane, invade into the inner membrane, and digest cellular components by different proteases. Then, they grow and replicate. After they finish multiple cycles for growth and replication, they lyse the prey and are released.

There are several kinds of predatory bacteria in different conditions. For example, predatory bacteria can grow in fresh water condition, soil condition, and even ocean condition. From those points, I became curious about what if predatory bacteria grown in normal water condition can predate as well as they can do in the in salt water condition, or which concentration of solution is critical to their predatory behavior.

There were some problems to fresh water BALOs in the salty solution. It was hard to find the tendency with molar or percent concentration. To solve this problem, I used osmolality which means total number of ions in solution, and it actually helps to solve the correlation problem. Also, we tested organic molecules able to show different effect or not. Therefore I used sugar and amino acids for this experiments.

To confirm whether this concept can apply to the natural condition, we used 'serum albumin' which is common in blood. Blood is one of salty condition in body. It is consist of multiple different molecules and proteins. For checking how BALOs can work in real situation, we tested it.

In this study, I wanted to show how well predatory bacteria can work in different salt condition, I used *Escherichia coli MG1655* pucdK which produce bioluminescent proteins as a main prey and *Bdellovibrio bacterivorous HD100* as a main predator, and to check whether this effect only happens to just *Bdellovibrio bacterivorus HD100* and specific prey species or not, I chose *Bdellovibrio bacteriovorus 109J* as another predatory bacteria, *Klebsiella pneumoniae* and *Acinetobacter baumannii* as additional prey.

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Chapter 1. BALOs and Environment

Introduction

1. Summary

BALOs, or *Bdellovibrio*-And-Like Organisms, are predatory bacteria. They predate other bacteria, normally Gram-negative bacteria. One of the major characteristics of BALOs is being ubiquitous which means they live numerous different conditions in nature. For example, they live in soil, ponds, lakes, rivers[1], the sea[2,3], and animal intestines, mainly upper intestine[4]. BALOs can be isolated from nature[5,6,7]. In this chapter, I will review papers from the other groups which deal with the way to isolate BALOs from the nature, and I will introduce which points are addressed in the thesis. The main paper is called ***Isolation and identification of Bdellovibrio and like organisms (BALOs) from various saltwater sites in the southern Cape Cod area, and analysis of their prey range specificity published on Microbial Diversity 2005 by Laura Hobley from Marine Biological Laboratory, University of Nottingham, UK[8].***

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Review

1. What are 'BALOs'?

People distinguish animals by what they eat. Herbivors only consume plants, carnivors only consume meat, and omnivors consume both. Bacteria can also be distinguished in this way. There are not only prey cells but also predatory cells. Predatory bacteria attack and kill prey bacteria. Among various bacterial predators, *Bdellovibrio*-And-Like-Organisms (so called BALOs) are especially well-known predatory bacteria. BALOs include *Bdellovibrio bacteriovorus* and similar bacteria such as *Bdellovibrio bacteriovorax*.

Bdellovibrio is a genus of Gram-negative, aerobic bacteria. As mentioned above, their well-known characteristics is that they parasitize other Gram-negative bacteria. *Bdellovibrio bacteriovorus* was first described by *B.bacteriovorus* Stolp and Petzold in 1962. There are other speices, *Bdellovibrio starii* and *Bdellovibrio stolpii*, which have been move to a separate genus Bacteriovorax[5,9].

BALOs exist in a broad range of environments and conditions[1,2,3,4,5,6,7]. For examples, they exist on the soil, in lakes, in sewages, and even in the ocean. In other words, BALOs can live anywhere if there are bacteria that can be used as a prey. They not only exist outside of the human body but also inside of the human body, especially in the upper intestine of human. BALOs are thus ubiquitous.

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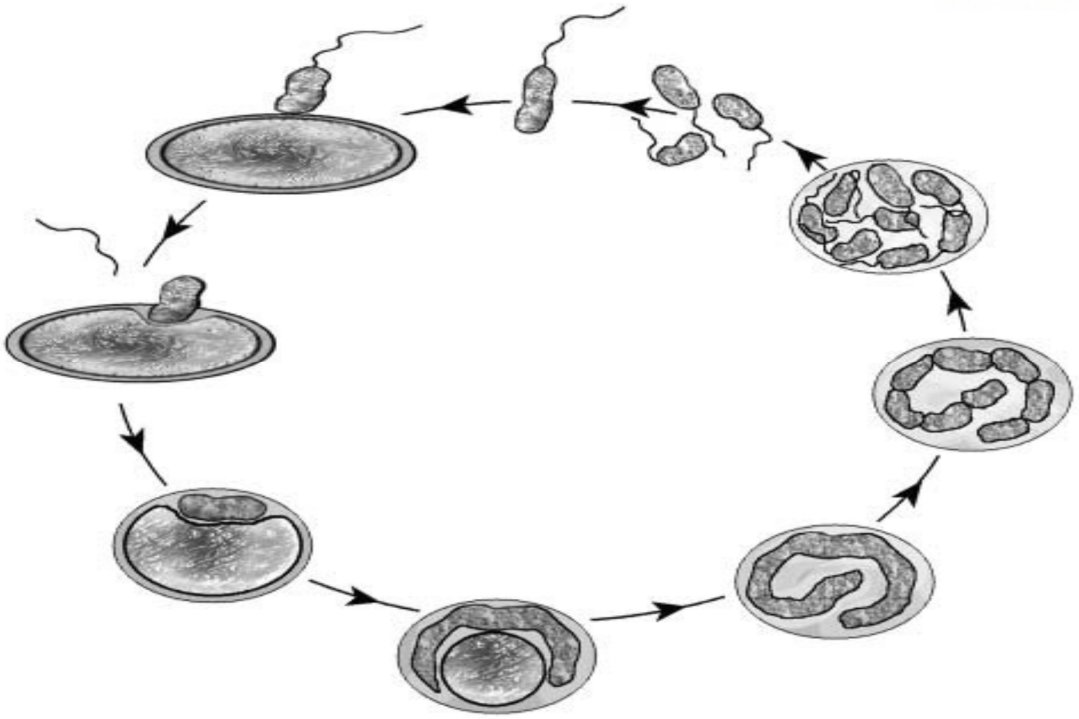

Figure 1. Life cycle of Bdellovibrio bacteriovorus. (Made by Megan E. Nu´n˜ ez, et al, 2003)[10]

2. Life Cycle of BALOs

BALOs have specific mechanisms to attack the prey.[9,10] BALOs have a flagella for swimming around and seeking a prey, and they attach themselves to the outer membrane and the peptidoglycan layer of prey. Then, they create small holes in the outer membrane of the prey. After attachment, the next step is invading the periplasmic space of the prey. Then, they reversibly attach to the prey cell for a short recognition period via the pole opposite the flagellum. After the recognition period, they irreversibly attach. Thereafter, they seal the membrane hole on the prey used for entering into the periplasmic space, and convert the host cell to a spheroblast. Afterward they digest and consume the cytoplasm of prey and replicate via many different types of hydrolytic enzymes. While they are predating prey, they form bdelloplast which is a complex of both predator BALOs and prey. Then, they start to grow until the nutrients inside of the host are exhausted. When there are not enough nutrients, the filaments separate to form progeny *Bdellovibrio*. After they predate the prey, they lyse the prey membrane, and then they look for another prey.

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3. How to isolate the BALOs

As BALOs are ubiquitous, so it is possible to find them in numerous different conditions and environments. One of the way to confirm there are BALOs is to isolate BALOs.

Firstly, the prey in the samples need to be isolated[6,8,11]. The BALOs have a prey range which means the kinds of prey that BALOs can predate. In the paper mentioned in the summary part, the method for isolation of the prey is introduced: The Samples have to be diluted like 100ul of sample, 10^{-1}, and 10^{-2}, and they were spread-plated on 1.5% agar plates, and single colonies were then picked and re-streaked at least 4 times to get pure isolates.

After isolation of prey, BALOs are isolated from the samples[6,7,8]. Non liquid samples have to be diluted with distilled water After vortexing the samples mixed with distilled water, they should be filtered with 0.45μm-filters. In the case of liquid samples, the samples do not need to be mixed with more water. The samples have to be filtered with 0.45μm-filters. After filtration with 0.45μm-filters, 100μL of filtered samples are mixed with top agar (5mL of 0.7% agar), and overnight-cultured prey (200μL). Then, after 2 or 3days, plaques are formed. Inoculation of BALOs from the plaques into the liquid media is performed with the same protocols such as filtration with 0.45μm-filters, addition of top agar and prey, growth on the plate, and inoculation into the liquid media to get purer BALOs, which can contain more than one strain of BALOs.

4. Prey Range Analysis

After isolation of BALOs, prey range analysis is necessary whether the isolated BALOs prefer specific prey[6,8]. BALOs predation rates depend on the strain. For example, *B.bacteriovorus* strains prefer to predate *E.coli*, but *Halobacteriovorax* prefer *Vibrio parahamolyticus*[12,13,14]. Therefore, prey preference is one of the clues to infer which species are contained in isolated BALOs. Finding the prey preference gives the hint to grow BALOs better.

Prey range analysis presents the possibilities for the application of isolated BALOs. For examples, if isolated BALO A can predate *Salmonella*, but isolated BALO B cannot predate *Salmonella*, then BALO A can be used for removing *Salmonella*. In this way, prey range analysis is one of the methods for finding the way to apply BALOs.

5. *Bdellovibrio* Clone Libraries

To confirm the exact species of BALOs isolated from the samples, comparison of DNAs from BALOs to DNA library is necessary[15,16]. DNA is the main difference that can be used to determine which species the isolated BALOs are. For comparison, first of all, DNA purification is necessary. The method for DNA purification of BALOs is the same as the normal DNA purification method for the

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Gram-negative bacteria. After DNA purification, PCR for a specific sequence of *Bdellovibrio* is following step for checking the species. In the case of primers, researchers used universal bacterial primers 8F and 1492-rev, along with the Bdellovibrio specific primers described in Snyder et al (Snyder et al, 2002)[17].

To track which kinds of BALOs exist in a sample, target genes are necessary. In the case of target genes, four enrichments were used in the preparation of *Bdellovibrio* clone libraries. [17] Several primers for enrichments were chosen to allow pairwise comparisons between *Bdellovibrio* spp from clams, and those from mussels along with comparisons between the preys used in the enrichments. The primers used in this step were mentioned above like the universal bacterial primers 8F and 1492-rev. PCR products are like Table 1 with each primer. The products containing more than one *Bdellovibrio* primers were combined and used in the preparation of the clone libraries. 24 sequences were obtained for each sample, and the results expressed as neighbor joining trees in figure 2 to 5

Forward primer	Reverse primer	Clams Prey1.4	Clams Prey1.5	G.Sipp Prey 6.2	G.Sipp Prey6.3
8F	1492rev	✔	✔	✔	✔
Bdello	1492rev	✔	✔	✔	✔
Bdello	bact	✘	✘	✘	✔
Bdello	starrii	✔	✔	✔	✔
Bdello	stolpii	✔	✔	✔	✘
Bdello	saltwater	✘	✘	✘	✘

Table 1. PCR primer combinations and positive PCR results from liquid enrichments from BALOs in Cape Cod area(Laura Hobley, 2005)[8]

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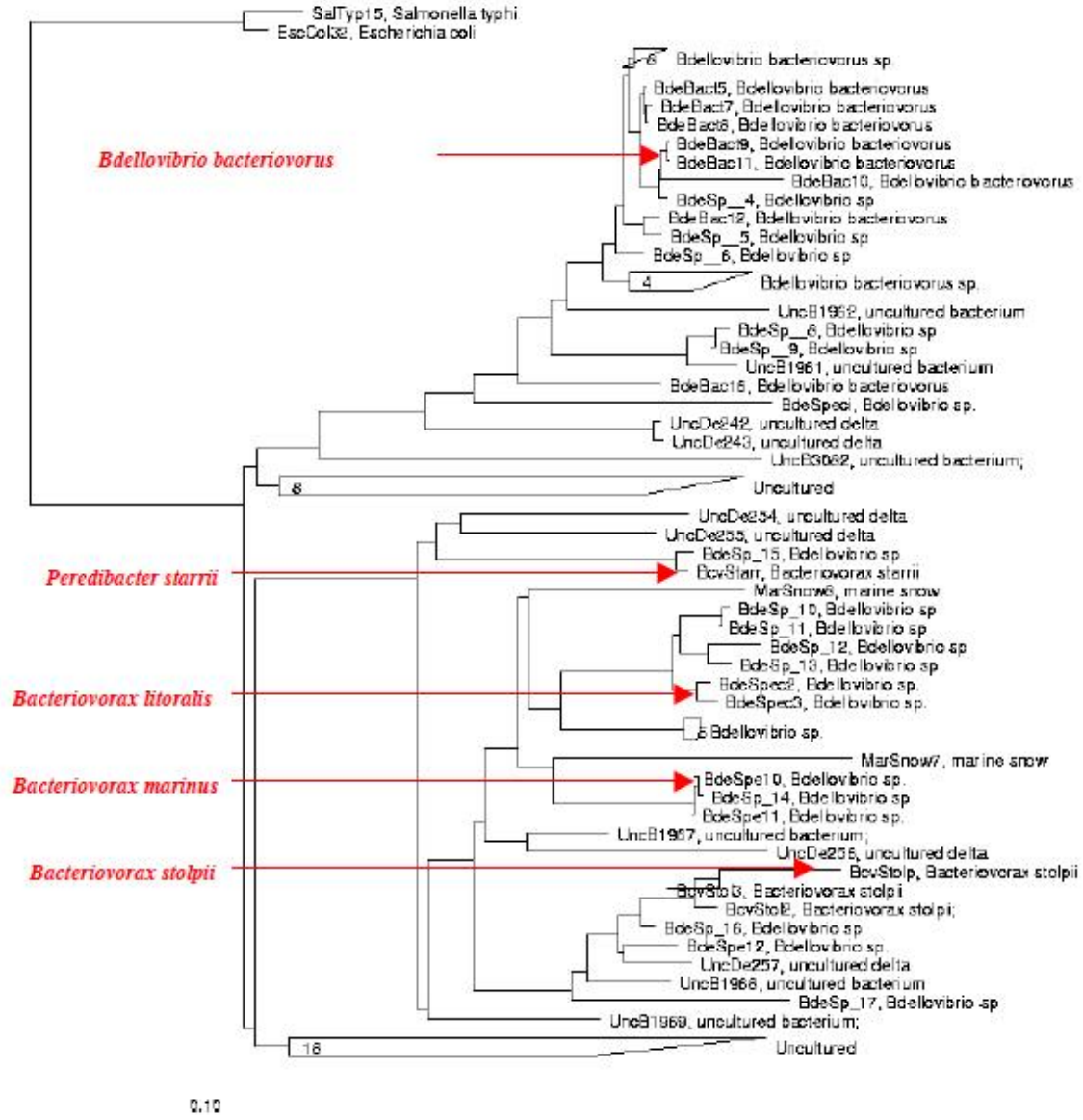

Figure 2. Neighbour joining tree of *Bdellovibrio* sequences present in the current ARB database (as at August 2005) (Laura Hobley, 2005)[8]

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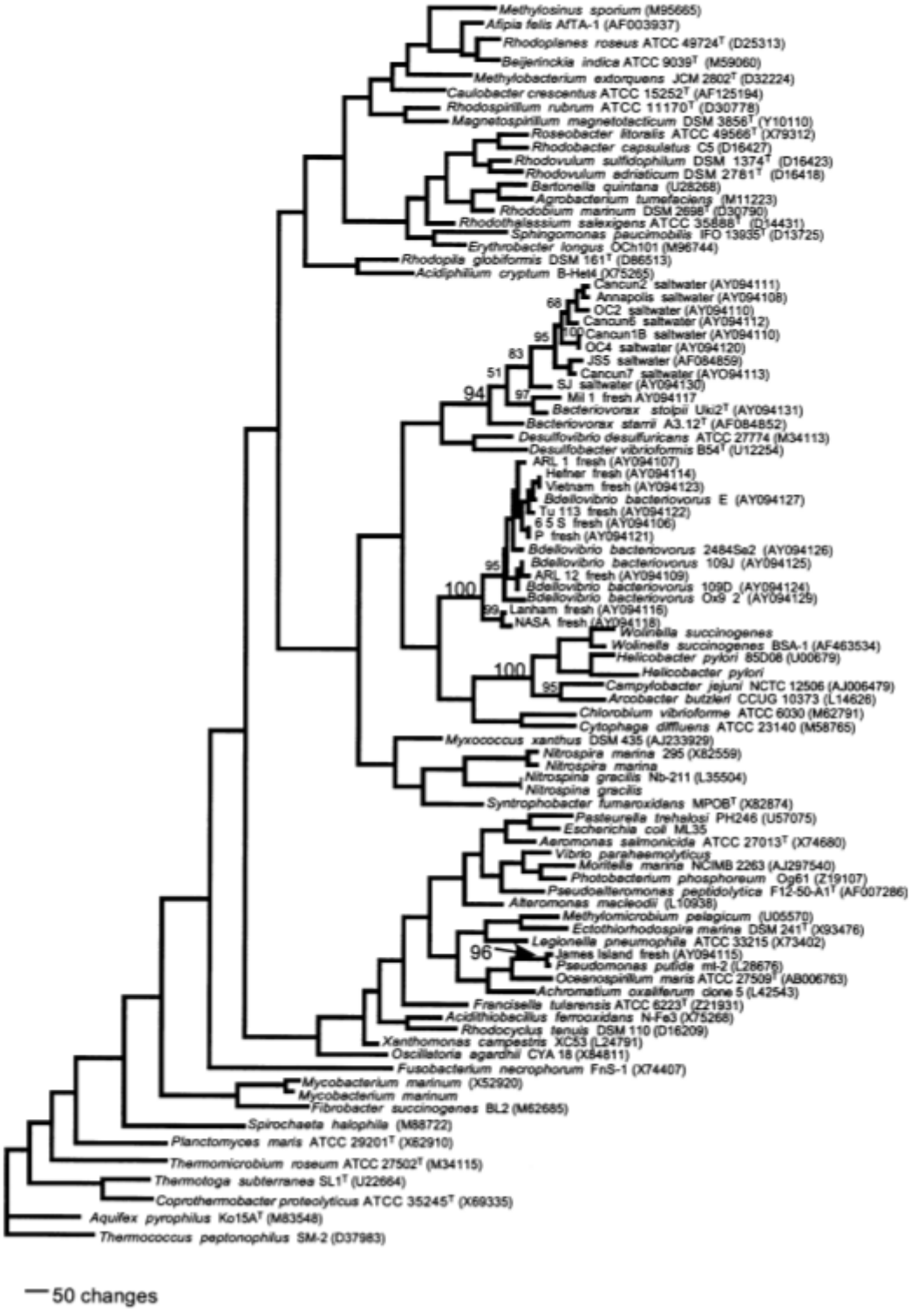

Figure 3. 1. Neighbour-joining tree of BALO isolates. A neighbour-joining tree was constructed for the 17 salt-water and nine fresh water isolates by a aligning these sequences with other selected members from prokaryotic domain (Andrew R. Snyder, et al, 2002)[18]

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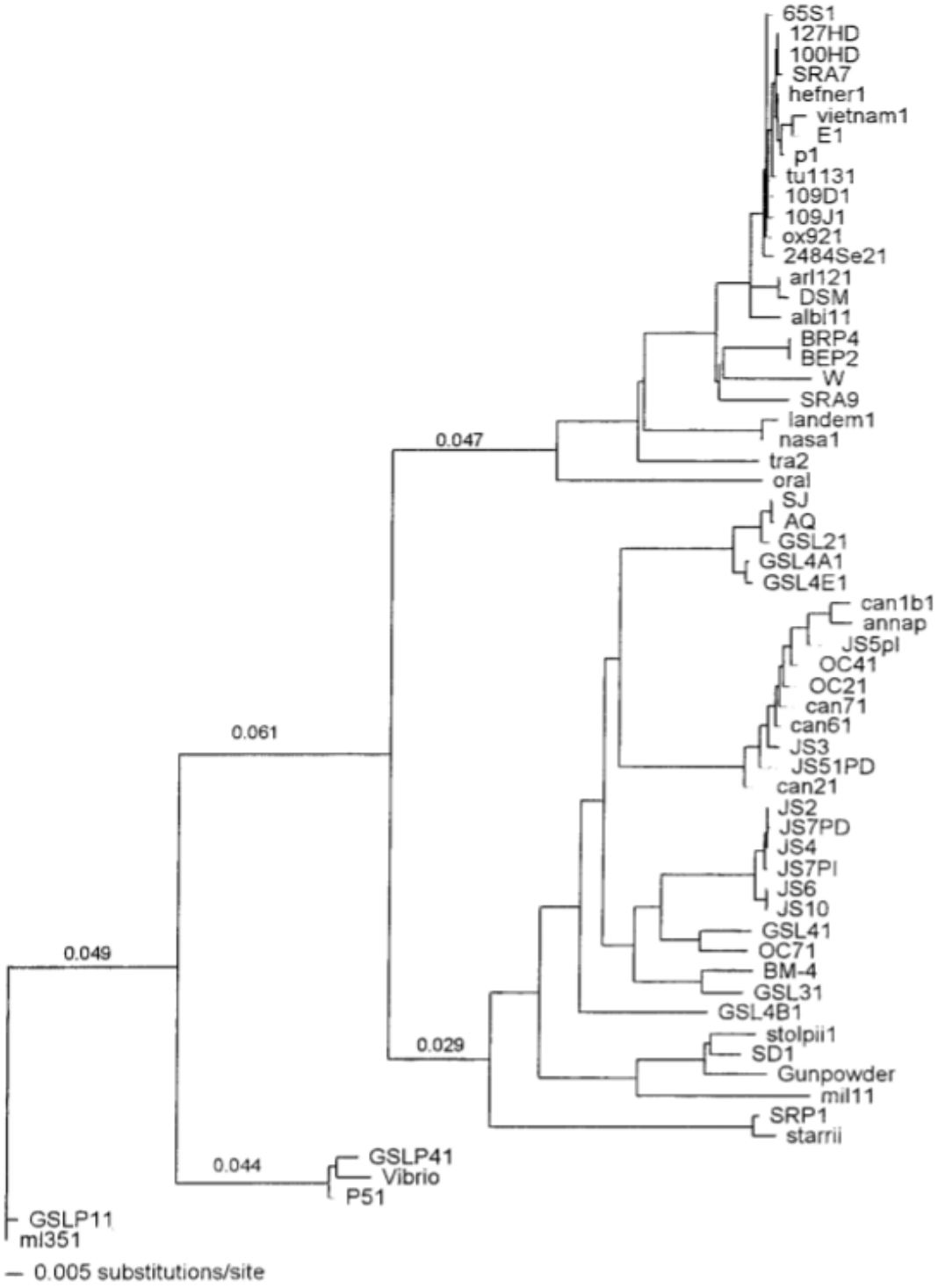

Figure 4. Phylogenetic tree of the Great Salt Lake predator isolates and other *Bdellovibrio vibrionaceae* (Silvia A. Pineiro, et al, 2003)[19]

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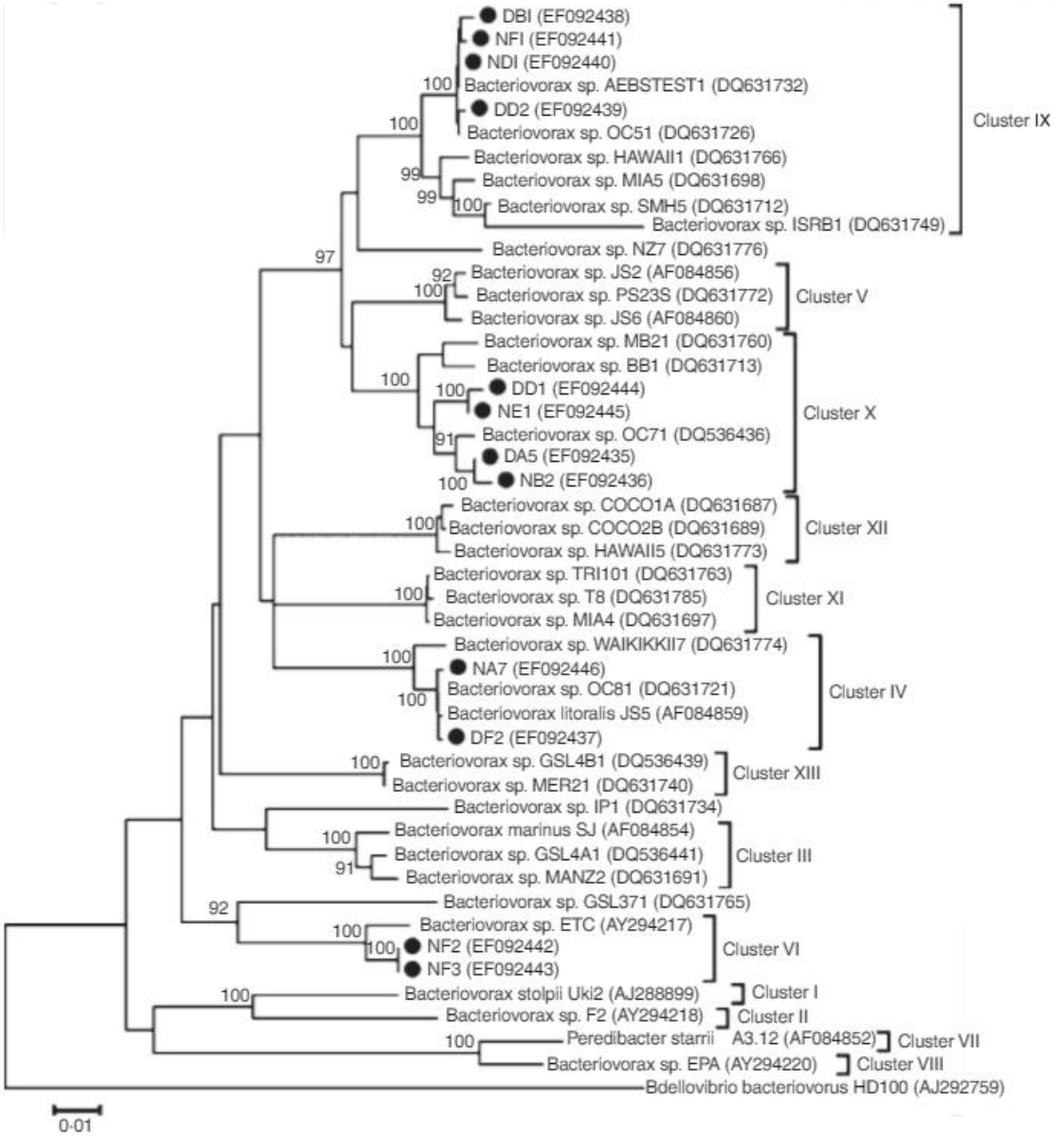

Figure 5. Phylogenetic tree of 16S rRNA gene sequences of *Bacteriovoracaceae* isolates from seawater shrimp ponds and adjacent coastal waters. (C.-Q.Wen, et al, 2009)[20]

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Conclusion

Depending on the position where the samples were, different species of BALOs were isolated. It seems like BALOs living in the river suddenly disappear along the river at a specific point, and different types of BALOs suddenly appears. In other worlds, there is one or more special factors that decide the exact species of BALOs to be able to conduct their lives to the suitable environment.

From isolation, identification and classification of BALOs, we can notice there are several different species of BALOs rivers and the sea. According to neighbor joining trees, BALOs living in the river are mainly *Bdellovibrio* bacteriovorus, but near the sea, isolated BALOs are mainly *Bacteriovorax* or different species of *Bdellovibrio bacteriovorus* In other words, BALO species differ depending on the environmental in which they are found.

Even though there is an influx of BALOs from the river to the sea, different BALOs exist at different sites along the same river. From this point, there is a question: What factors determine differentiation among BALO species? My hypothesis is salt concentration of the environment BALOs live affects the BALOs, so the specific BALOs exist in different environments following their preference. Therefore, in the following chapter, we will test about which factor can affect BALOs preference.

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Chapter 2. Osmolality effect on *Bdellovibrio bacteriovorus*

Introduction

1. Summary

Bdellovibrio-And-Like-Organisms, BALOs are sensitive to multiple environmental factors. It means that there are numerous factors affecting BALOs activities[21,22,23]; for example, temperature and pH are kinds of major factors. The pH and temperature cannot make the differences between BALOs living in the river and the sea. Thus, we could ask how does saltwater affect BALOs from the river. To understand this, we did series of experiments with *Bdellovibrio bacteriovorus* into the several different solution with a specified factor of the river and the sea, osmolality

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2. Ubiquitousness of *Bdellovibrio bacteriovorus*

We had a question about the ubiquitousness of *Bdellovibrio bacteriovorus* and similar BALOs: which factors make differences between the BALOs from the river and the BALOs from the sea. Among several factors, we thought that osmolality is the main factor behind the differences. Osmolality is the important aspect of the solution, and according to previous research, protein structures also are affected by osmotic pressure. From this point of this view, this chapter shows how osmolality affects the predation of BALOs in different molecular solutions.

3. What is 'Osmolality'?

Osmolality is the index for osmotic pressure. If molecules dissolve into solvent, molecules float around the solvent. While the solute molecules are floating, they interact with the solvent molecules and other solute molecules. As a result, there are repulsion and attraction between molecules. Those things make the osmotic pressure of a solution. There are two units that express the osmotic pressure per unit square; one is 'osmolarity', and the other one is 'osmolality'. The former means osmoles per unit liter of solution while the latter means osmoles per unit kilogram of the solvent. (Osmole is the unit that represents how many ions dissolve in a solution. For example, when 1mol of NaCl dissolve into 1L of water, that NaCl solution has 2osmol.) Osmoalarity and osmolality sound similar, but osmolality is not influenced by temperature and pressure because it is based on the weight of the solvent. It expresses electrolyte-water balance of the solution. Its unit is based on the osmole and the kilogram, so it does not change following changes in temperature and in pressure. Therefore, it is an accurate and steady value. In contrast with osmolality, osmolarity is based on the volume of the solution. As a result, osmolarity is variable depending on the temperature and pressure. Osmolality is hard to calculate, but it has a special tools to measure. It is an osmometer. In this experiment, we used the osmometer 'Model 3320' from '*Advanced Instruments*, *INC.*'.

There are several types of osmometer such as freezing point depression osmometer, and vapor pressure depression osmometer. When a solute dissolves into a solvent, both solute and solvent attracts each other. Consequentially, the freezing-point decreases, and the vapor pressure decreases. The osmometers measure how much the freezing-point and the vapor pressure decreases, and they convert the result as an osmolality. The osmometer mentioned above and used in this experiment is a freezing point depression case.

In following experiments, we used osmolality as an index for osmotic pressure. The reason

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why we used osmolality for this study is that we tried several different salts such as NaCl, KCl, $MgCl_2$, and $CaCl_2$ with different molarity or percentage, but there is not clear correlation between results and molar concentration. That means we needed to find another indicator that shows solution characteristics. The conclusion about the new indicator was osmolality. For almost all inorganic salt solution, they show similar tendencies; predation occurs around 150mOsm/kg, and over 150mom/kg, predation can occur or not depending on the molecules. That means, osmolality is a more suitable indicator for inorganic solutions.

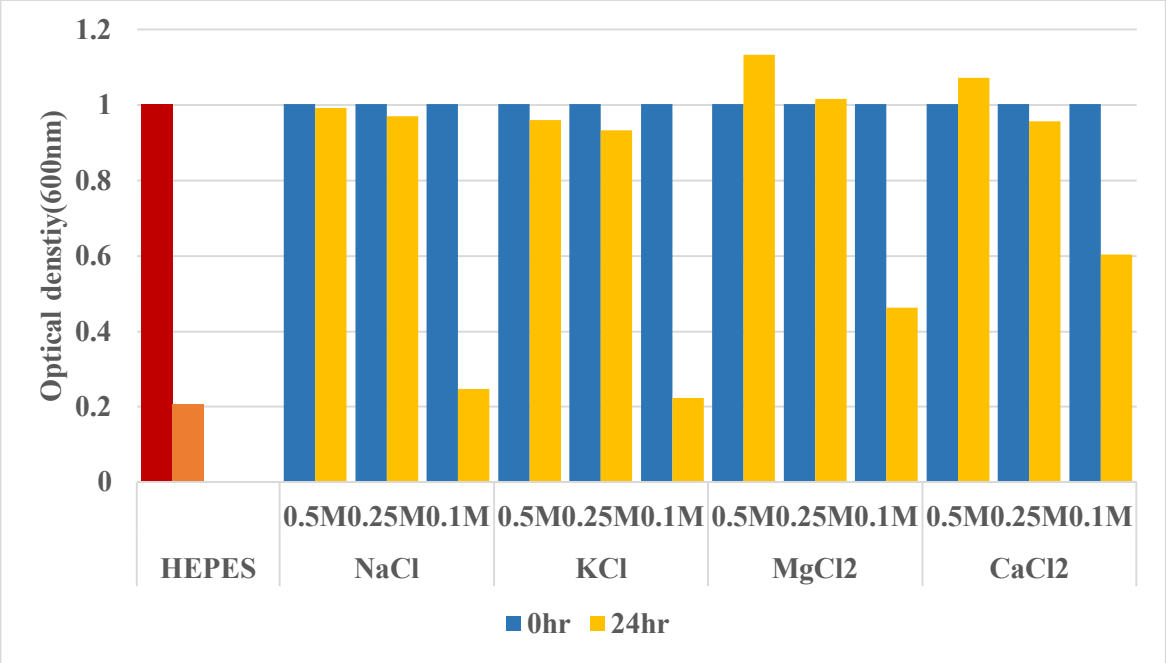

Figure 1. Predation of *B.bacteriovorus* in different molar concentration of different salt solution.

This test is for finding any co-relationship between different inorganic compound solutions with different molar concentration. This test is done with *E.coli* and *Bdellovibrio bacteriovorus* HD100. In the case of all 0.5M and 0.25M samples, there is no predation, and 0.25M is around the molar concentration of sea water. However, in the case of 0.1M $CaCl_2$ solution, it is hard to say there is predation. Also, in the case of 0.1M of each salt solution, NaCl and KCl show similar tendency, but $MgCl_2$ and $CaCl_2$ show different tendency from previous two salt cases. Moreover, both 0.1M of $MgCl_2$ and $CaCl_2$ show very similar tendency. As a result, we can say same molar concentration of different salt solution have different impact on *Bdellovibrio bacteriovorus* HD100 predation, so molar concentration does not show a clear co-relationship.

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4. Purpose of this study

The purpose of this study is to confirm *Bdellovibrio bacteriovorus* ability to prey in a high osmotic condition. Recently, BALOs are raised as possible candidates for alternative antibiotics[26,27,28]. Humans are now facing the problem of antibiotic-resistant bacteria derived from abusing and overusing antibiotics. Abuse and overuse of antibiotics cause super-bacteria: the bacteria that have a resistance to multiple antibiotics. In other words, super-bacteria is hard to treat with antibiotics already used. From that point of view, BALOs have risen as a candidate to cure super-bacteria. *Bdellovibrio bacteriovorus* applied as an alternative antibiotics is one of the ultimate goals for BALO study. Human bodies are full of multiple different types of liquid. Those types of liquid are distinguishable from fresh water. In other words, they have many different possible factors that interrupt BALOs from predating preys. Therefore, through this study, we can check if *Bdellovibrio bacteriovorus* are able to be active in the human body.

There are several interruptible factors against BALOs predation such as viscosity, pH, and osmolality. Viscosity disturbs BALOs swimming with flagella, and pH deforms proteases from BALOs for predating prey components. Viscosity and pH must be the same because there is no difference which makes viscosity and pH. Therefore, osmolality also gives BALOs obstacles for predation in the way of causing changes in proteases, osmotic pressure between inside and outside of BALOs, and destruction of ion balance inside of BALOs and preys. Like this, osmolality acts as one of the major effects in the predation of BALOs. Therefore, we need to check how osmolality influences BALO, and whether organic compound solutions also give the same impact.

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5. Materials and Method

1. Materials

1-1. Media[9,29,30,31,]

Luria-Bertani broth

All prey was grown in Difco™ Luria-Bertani broth, Miller from 'Becton, Dickson and Company'. The composition of Luria-Bertani media is tryptone 10.0g, yeast extract 5.0g, and sodium chloride 10.0g per a liter. Media are always autoclaved at 121℃ for 15minutes. The final pH of LB media is 7.0±0.2. All Colony-Forming unit (CFU, confirmation of the number of bacteria) of prey were checked on the LB plate with micro agar from Accumedia™ (the percentage agar is 1.7%).

HEPES

All BALOs were grown in buffer media HEPES, N-(2-Hydorxymethyl) piperazine-N`-(2-ethanesulfonic acid) from Sigma Aldrich. Its molecular formula is $C_8H_{18}N_2O_4S$, and its molecular weight is 238.30g/mol. The final concentration of HEPES is 25mM mixed with BALO salt (3mM $MgCl_2$ and 2mM $CaCl_2$). The pH of HEPES was adjusted with pH 7.2.

DNB

To check the number of BALOs after predation, we did PFU (Plaque Forming Unit). For PFU, we used DNB plates. DNB is the acronym of 'Diluted Nutrient Broth'. Its composition is beef extract 3g, enzymatic digest of gelatin 5g per a liter. Its final pH is 6.8±0.2. If we make plate with DNB for check PFU, we made 1.7% plate as a bottom agar.

Filter

To use BALOs from the media, we need to filter it not to add prey. To separate BALOs from prey, we used 0.45μm filters from Merck Millipore. The filters are made with PES membrane, and they are sterile.

Protease Activity Assay kit

One possible reason why osmolality affect BALOs activity is that osmolality influences proteases activity from BALOs which is necessary for BALOs to attack and consume prey. Therefore, we needed to check it with protease activity assay kit. The protease activity assay kit is produced by Abcam. Kit is consist of assay buffer 25mL, protease substrate (lyophilized) 1 vial, FITC standard (25μM) 200μL, and Positive Control (lyophilized) 1 vial. The protease substrate is reconstitute with 220μl dH_2O. We pipette up and down to completely dissolve. The positive control is reconstitute with 100μl Assay Buffer. We pipette up and down to completely dissolve.

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1-2. Osmometer

To make solution with appropriate osmolality, we used osmometer to check osmolality of solution. The osmometer was produced by Advance Instruments, Inc. This osmometer is a freezing point depression osmometer. Its maximum detectable value is around 1500mOsm/kg.

1-3. Solute, and Solution

All solutions were made in deionized water. In this study, there are two types of solutes to compare the consequence. One is a set of inorganic compounds such as sodium chloride, potassium chloride, magnesium chloride, and calcium chloride. The other one is a set of organic compounds which contains two types; one is sugar, and the other one is amino acid. For sugar, we used xylose and sucrose, and for amino acid, we used tryptone.

1-4. Cell

BALOs are predatory bacteria, so we need to prepare prey cell to feed them up to grow. In following experiments, we have used two sorts of *Bdellovibrio bacteriiovorus* such as *Bdellovibrio bacteriovorus* HD100, and *Bdellovibrio bacteriovorus* 109J, and three sorts of preys such as *Escherichia coli* MG1655, *Klebsiella pneumonia*, and *Acinetobacter baumannii* clinical isolation 1. For sugar and amino acid tests, we used special *E.coli* which has mutation in diaminopimelic acid (as known as DAP) synthesis, *E.coli* DAP^- strain. The reason is that *E.coli* MG1655 pucdK can consume sugar and amino acids, so it drops pH which is negative to *Bdellovibrio bacteriovorus*.[50]

2. Methods

2-1. Prepare prey and BALOs

To prepare BALOs for the experiment, we need to prepare both prey and the predator. In the case of prey, we used Luria-Bertani(LB) broth. To feed the BALOs, firstly, we grow preys into liquid LB broth for 24 hours at 37℃ in shaking incubator (230rpm). After 24 hours, we centrifuge down prey cells with 2000 to 3000rcf at 4℃ for 15 minutes. Then, we discard the supernatant and resuspend the pellet with HEPES buffer(pH 7.2, 25mM) to make prey OD(optical density)1.0. Into this prey-HEPES mixture, we add the BALOs filtered by 0.45μm filter with 10μL per 1ml of prey. After addition of BALOs into prey-HEPES mixture, we keep them at 30℃ in shaking incubator with 230rpm for 24 hours. All of the tests use only *E.coli* MG1655 predating BALOs.

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2-2. Preparation of solutions

First of all, we need to prepare the salt solutions. We made 5M to 1M of solutions and check the osmolarity of serially diluted solution with osmometer. The osmometer measures the osmolality of solutions. In this step, we optimize molar concentration and osmolarity relationship graphs. After optimization of molar concentration-osmolality graph, we ascertain the buffer system which we use for BALO predation to prevent higher osmolarity than the solutions we need. For example, in the case of HEPES(pH 7.2, 25℃, 25mM), it has 40mOsm/kg. Therefore we made a solution with 2X solution subtracted 40mOsm/kg compared to which we desired. The reason why we need to make 2X solution is to mix the solution with resuspended pellet of 24hour-grown prey with OD(optical density) 2.0 at the ratio of one-to-one by HEPES buffer. There are two ways to prepare salt solution-prey mixture. First one is that we prepare the prey with OD1.0 by HEPES buffer and centrifuge down the prey, then we resuspend it with the designated osmolality solution. The other one is that we prepare the prey with OD2.0 by HEPES buffer, then we mix with prepared doubled-osmolality solution. We prefer to use second method. Before addition of BALOs, but after mixing prey and BALOs, we need to check optical density of prey-solution-HEPES mixture to confirm whether its OD is 1.0.

2-3. Experiment method

CFU (Colony-Forming Unit)

After preparation of doubled-osmolality salt solutions and OD 2.0 preys, we mix a salt solution with a prey at one-to-one ratio. For example, if we want to make 300mOsm/kg salt solution-prey mixture, we prepare 460mOsm/kg salt solution (because HEPES has 40mOsm/kg osmolality which we use for BALO-activity buffer) and OD2.0 prey, and we mix both together one-to-one ratio until it becomes 4mL. After of it, 40μL of 0.45μm-filtered BALOs is added to 4mL (1μL of BALOs per 100μL of prey). Then, they grow in 30℃ shaking incubator with 230rpm for 24hours. After 24hours from adding BALOs, we dilute the sample, and we spread on the LB plate (1.7% micro agar).

PFU (Plaque-Forming Unit)

After preparation of doubled-osmolality salt solutions and OD 2.0 preys, we mix a salt solution with a prey at one-to-one ratio up to 4mL. In that prey-solution mixture, we add 40μL of filtered BALOs. Then, we grow them at 30℃ shaking incubator with 230rpm for 24hours. After 24hours, we dilute samples and add diluted BALOs into 15mL tube. Then, we add 2mL of 24hour-grown prey 2ml and 6mL of top agar (DNB with 0.7% micro agar) into BALO-added 15mL tube. After all, we invert the tube slowly to mix prey, top agar, and BALOs, and we pour it on the DNB plate (1.7% micro agar, bottom agar).

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Azo dye impregnated collagen (Azocoll)

Azo dyes impregnated collagen, azocoll is an insoluble protein-dye conjugate, and it is used widely for the assay of proteolytic enzymes[44,45]. Azocoll is hydrolyzed readily by a variety of proteinases and yields soluble, colored peptide in proportion to enzyme concentrations. Azocoll was purchased from Sigma Aldrich. The method for proteolytic enzyme assay is done as following: we grow *Bdellovibrio bacteriovorus* HD100 with HEPES and 300mOsm/kg under 5X Nutrient Broth condition. After 24 hours, we filter those *Bdellovibirio bacteriovorus* HD100 with 0.22μm filter. Then, we add 15mg of azocoll in 4mL of filtered *Bdellovibrio bacteriovorus* HD100. Additionally, we take absorbance 525nm with TECAN Magellan™.

Protease activity assay kit

To confirm protease activity, we used protease activity assay kit produced by Abcam. We prepare test samples up to 50μl/well with Assay Buffer in a 96-well plate. For positive control, we use 5μL of the reconstituted Positive Control solution into wells, and we adjust volume to 50μL with Assay Buffer. A reagent background control is 50μL of Assay Buffer. Also, we have to make standard curve with the known sample, so we add 0, 2, 4, 6, 8, 10μl FITC Standard into a series of standards wells. Then, we adjust the final volume to 100μl/well with Assay Buffer to generate 0, 0.05, 0.1, 0.15 0.2, and 0.25 nmol/well of the FITC Standard. After all, we make reaction mix. To do it, we mix enough reagents for the number of assays to be performed. For each well, we prepare a total 50μL Reaction Mix: 48μL for Assay Buffer, 2μL for Protease Substrate Solution. We add 50μL of the Reaction Mix to each well containing the Positive Controls, Reagent Background Control and Samples. For measurements, we use 485/530 nm fluorescence after we incubate it at 25℃ for more than 30 minutes.

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Results

1. Predation of *Bdellovibrio bacteriovorus* HD100 in NaCl solution against *E.coli*

As the preliminary test, we test with NaCl solution first. Firstly, we make a standard graph of the relation between Osmolality and molar concentration (25℃, 1atm). We make 1M NaCl solution, and do serial dilution to make the graph. After that, we measure osmolality with osmometer. The results are on Table 1 and Figure 1.

	Sample 1	sample 2	sample 3	sample 4	sample 5	average
1M	1788	1784	1784	1792	1774	1784.4
0.5M	896	895	890	881	885	889.4
0.25M	431	425	428	426	426	427.2
0.125M	227	228	225	225	226	226.2
0.0625M	113	114	114	114	114	113.8

Table 1. Real measured value of each molar concentration to osmolality (25℃, 1atm)

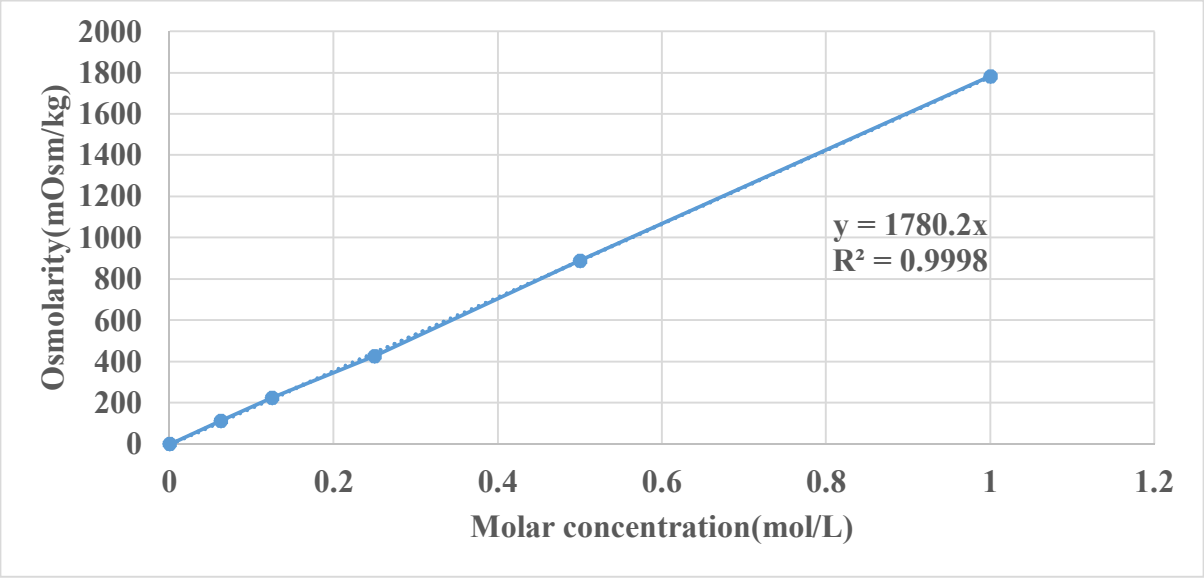

Figure 2. Osmolality-Molar concentration relationship

After confirmation of relation between molar concentration and osmolality, we checked the effect on osmolality on *Bdellovibrio bacteriovorus* HD100 predation. First of all, we checked optical density changes after addition of *Bdellovibrio bacteriovorus* HD100. Figure 3 is the optical density data.

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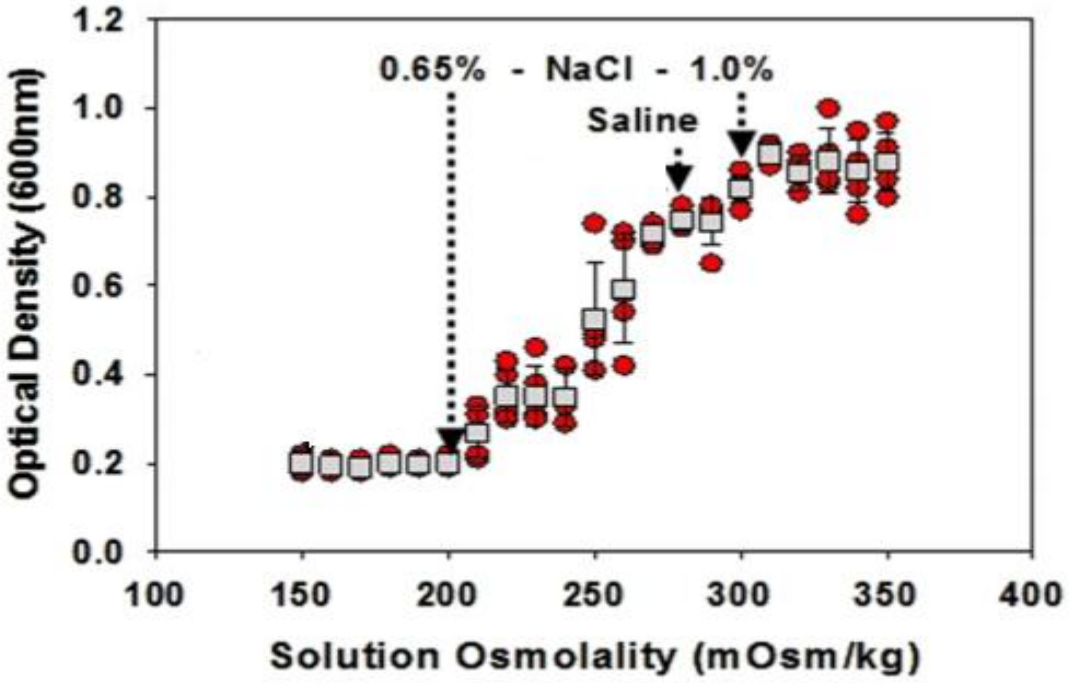

Figure 3. Osmolality of NaCl and optical density (600nm) (n=3)

According to figure 3, there are two boundaries in 200mOsm/kg and 300mOsm/kg. From 200mOsm/kg to 300mOsm/kg, predation activities of *Bdellovibrio bacteriovorus* have the problem with osmotic effect from surrounding solution molecules. 200msom/kg NaCl solution is approximately around 0.65% NaCl solution, and 300mOsm/kg NaCl solution is around 1.0% NaCl solution. To confirm precise impacts on *Bdellovibrio bacteriovorus* HD100 predation, CFU data is shown on figure 4.

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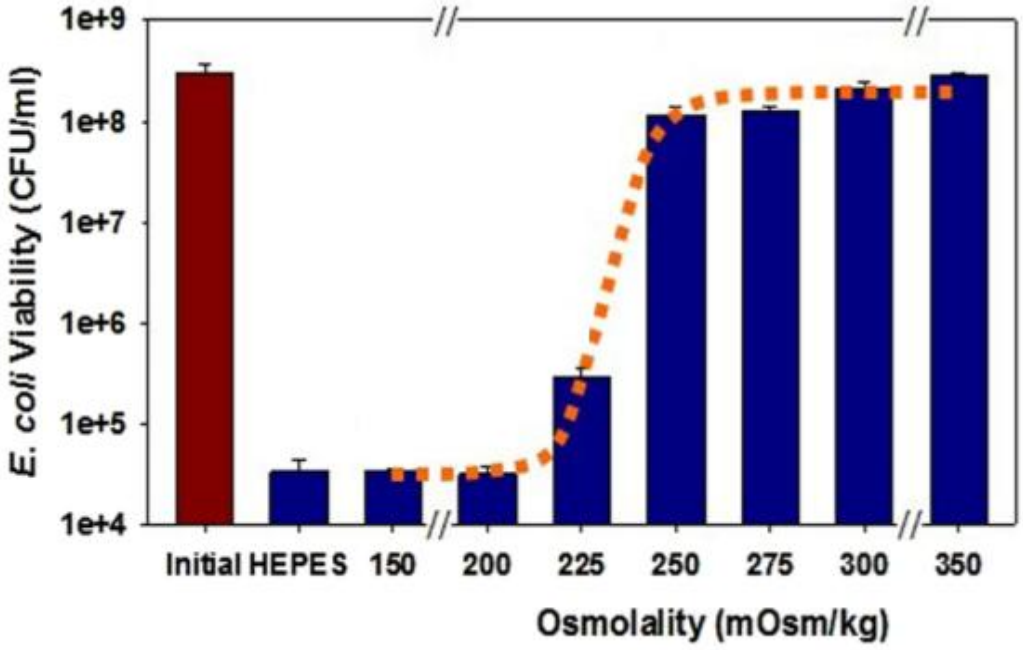

Figure 4. *E.coli* viability after 24hour-HD100-predation under different osmolality of NaCl (n=3)

According to prey viability data, at the osmolality lower than 200mOsm/kg, *Bdellovibrio Bacteriovorus* HD100 can show their predation ability against *E.coli* MG1655, but at the osmolality higher than 250mOsm/kg, it is interrupted from osmotic effects. However, between 200mOsm/kg and 250mOsm/kg of NaCl solution, there is decrease in BALOs' predation ability. By unknown mechanisms, *Bdellovibrio Bacteriovorus* HD100 is suffered in the range between 200mOsm/kg and 250mOsm/kg. *Bdellovibrio Bacteriovorus* HD100 is sensitive to high osmotic pressure.

There are several possible hypothesis for this phenomenon: high osmolality kills the preys, interrupts attacking abilities of *Bdellovibrio Bacteriovorus* HD100, blocks life cycle of *Bdellovibrio Bacteriovorus* HD100(Bdelloplast viability), and inhibits its protein functions. To figure out each possible reason, several experiments are required. One is test of checking attacking abilities for *Bdellovibrio Bacteriovorus* HD100, other one is test of bdelloplast viability test, and the last one is test with protease activity assay kits to check protease activity from *Bdellovibrio Bacteriovorus* HD100.

The test for confirming attacking ability of *Bdellovibrio Bacteriovorus* HD100 is to observe how much of preys has been predated for a short term, three hours. The method has multiple steps. Adjustment of the ratio between prey and predator one to one (PPR; prey-predator-ratio) is first step. To adjust PPR 1, centrifuging down the prey *E.coli* Mg1655 pucdK with 3000rcf for 15minutes at 4℃ is the first step, and then pellet should be resuspended with HEPES buffer to make OD3.0. At the same

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time, we centrifuge down the predator *Bdellovibrio bacteriovorus* HD100 with 5000rcf for 20minutes at 4℃(because at OD1.0, the number of *E.Coli* is about $1x10^9$, and the number of doubly concentrated *Bdellovibrio bacteriovorus* HD100 is about $1x10^9$). To make the solution, the different osmolality of NaCl solutions and HEPES are mixed into 3.8mL with one-to-one ratio. In mixed NaCl-HEPES solution 3.8mL, 100μL of each prepared prey and predator is added. Three hours later after mixing of solution with prey and predator, CFU of mixture is checked. The CFU data is shown on figure 5.

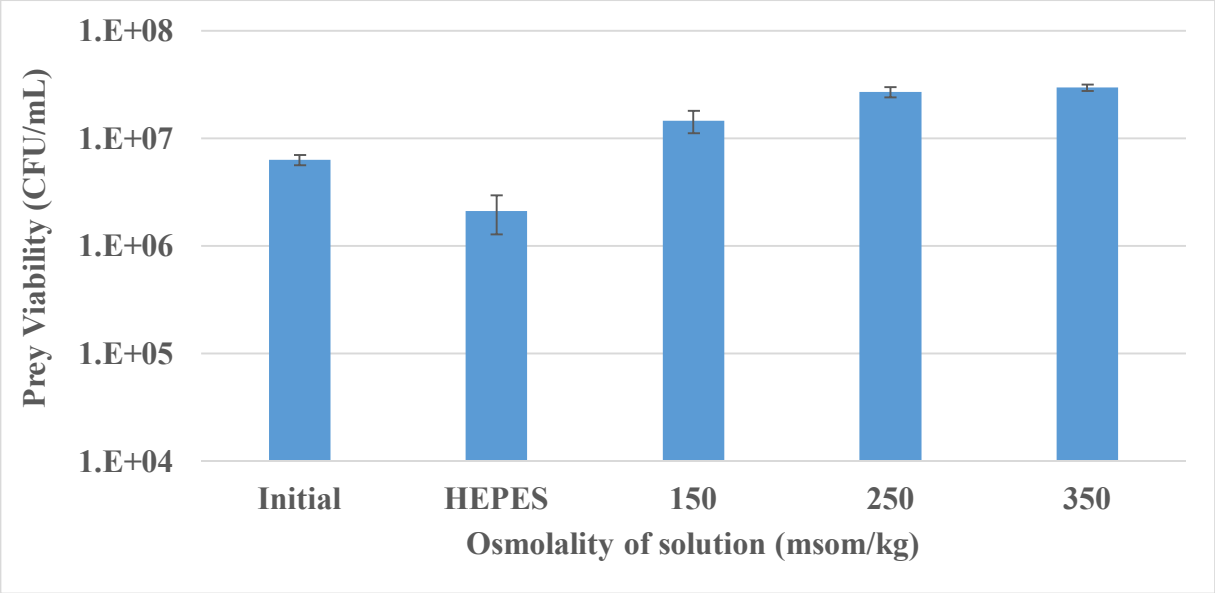

Figure 5. *Bdellovibrio bacteriovorus* HD100 attack ability confirming Test in Different Osmolality of NaCl solution (n=3)

If there is not any problems, then the prey viability (the number of CFU/mL) should decrease. However, if you compare 0hr to 150mOsm/kg, 250mOsm/kg and 350mOsm/kg, even though there is predation under 150mOsm/kg of NaCl solution, there is interruption to *Bdellovibrio bacteriovorus* HD100 in attacking ability for short term. That is, osmotic pressure derived by osmolality of NaCl solution affects to early stage of predation.

In the case of BALOs attack phase, bdelloplast forming step is one of the most important parts to *Bdellovibrio bacteriovorus* HD100 for proliferating. If you see figure 5, *Bdellovibrio bacteriovorus* HD100 can be affected by osmotic pressure critically in short term predation. To figure out from which step *Bdellovibrio bacteriovorus* HD100 are affected by osmotic pressure, checking bdelloplast is the mandatory test.

Bdelloplast is the complex of *Bdellovibrio bacteriovorus* and their preys. It is the basic step for predation activities of *Bdellovibrio bacteriovorus*. In other words, when the *Bdellovibrio bacteriovorus* has troubles in bdelloplast formation and growth, *Bdellovibrio bacteriovorus* is not able to predate the

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preys with the normal rate. If there is a problem to *Bdellovibrio bacteriovorus*, then the number of *Bdellovibrio bacteriovorus* decrease.

To check the condition of Bdelloplast, we checked bdelloplast viability after six hours from *Bdellovibrio bacteriovorus* forming bdelloplast. Bdelloplast growth confirmation test has series of processes. First of all, we prepare the number of both prey *E.coli* MG1655 pucdK and predator *Bdellovibrio bacteriovorus* HD100 to become one-to-one ratio. The method to adjust the ratio between the preys and the predators (PPR 1) is mentioned above. After preparation of the prey and the predator mixture, the mixture of preys and predators with PPR 1 is incubated for an hour to induce bdelloplast forming. Then, the one-hour-incubated mixture is centrifuged with 2000rcf for five minutes to mix with NaCl solution. Therefore, after centrifugation, of mixture, it is resuspended with half of total volume that is centrifuged. Following this, resuspended mixture is mixed with NaCl solution that has twice higher osmolality than actual desires because it is mixed one-to-one. Afterward NaCl-bdelloplast mixture is incubated for 6hr, and PFU of it is checked. The result of this experiment is shown on figure 6.

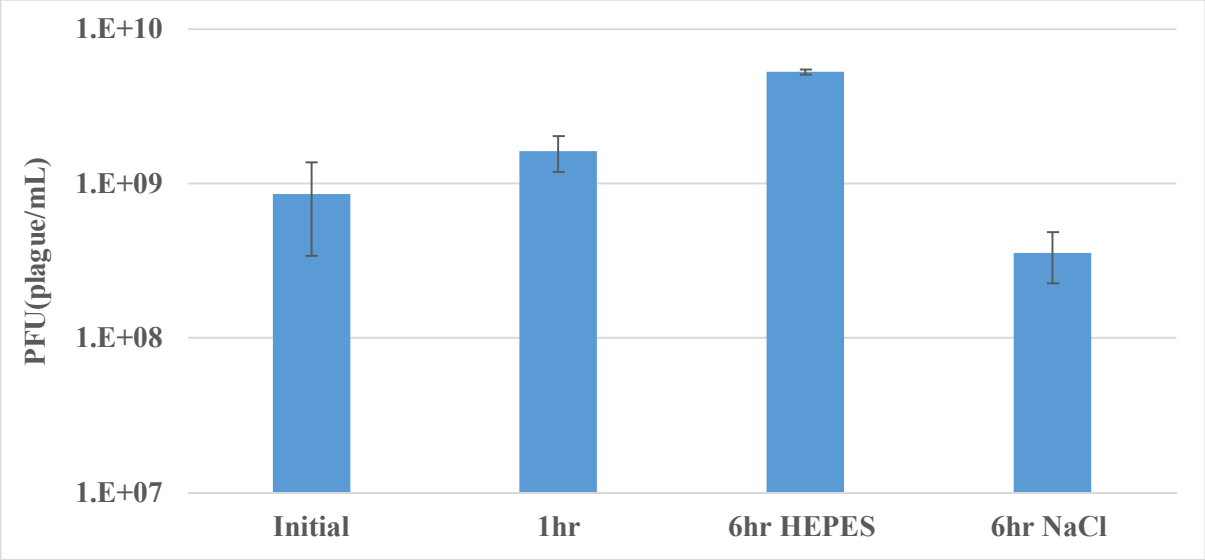

Figure 6. Bdelloplast growth confirmation under 350msom/kg of NaCl solution (n=3)

If we see the Figure 6, the number of plague in 0hr sample and 6hr HEPES sample have about five times difference, but if we compare the number of plague in 0hr sample and 6hr NaCl sample, the number of plague of 6hr NaCl case decrease about 60% of 0hr sample. That means, osmotic pressure interrupts bdelloplast viability.

The possible hypothesis why *Bdellovibrio bacteriovorus* HD100 is affected by osmotic pressure can be hypothesized in terms of their methods to predate other bacteria. Their main tools for predation are multiple different types of proteases[32]. The table 2 introduces significant proteases and peptidases

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used for predating in host-independent *Bdellovibrio bacteriovorus* HD100 (Derived from *Bdellovibrio bacteriovorus* HD100, but it does not necessarily predate other bacteria. That means, it consumes nutrient in the broth. However, it can produce almost all proteases that host-dependent *Bdellovibrio bacteriovorus* HD100[32]). As we can see in the Table2, there are multiple sorts of proteases and peptidases used by *Bdellovibrio bacteriovorus* HD100 for predation. According to the paper published in 2016 by Mark S. Miller et al[34], amino acids and peptides can be affected by osmotic pressure. That means protein itself can be affected by osmotic pressure as well. Therefore, we checked the protein activities under high osmolality condition which affect the BALOs' predation activities.

Gene	Uniprot Accession	Protein Name	Size (kDa)	Subcellular Localization
Proteases				
Bd2269	Q6MKV8	Serine protease, subtilase family	56.6	Extracellular
Bd2675	Q6MJU3	Putative membrane protein with protease subunit	33.5	Unknown
Bd2321	Q6MKR4	Subtilisin-like serine protease	74.9	Unknown
Bd2428	Q6MKG5	Serine protease	114.9	Extracellular
Bd1444	Q6MN19	Serine protease, subtilase family	111.5	Unknown
Bd2692	Q6MJS6	Protease	53	Extracellular
Bd2627	Q6MJY9	Periplasmic protease	32.3	Unknown
Bd2535	Q6MK75	Putative serine protease	28.5	Unknown
Bd3857	Q6MGR5	Alkaline serine protease subtilase family	43.2	Extracellular
Bd0449	Q6MQL6	Putative protease	57.2	Unknown
Bd2675	Q6MJU3	Putative membrane protein with protease subunit	33.5	Unknown
Peptidases				
Bd0306	Q6MQZ5	Carboxypeptidase	34	Unknown
Bd1962	Q6MLP5	Putative V8-like Glu-specific endopeptidase	31.3	Unknown
Bd3622	Q6MHC8	Aminopeptidase	45.6	Extracellular
cpt	Q6MIC9	Carboxypeptidase		N/A
dcp	Q6MII6	Peptidyl-dipeptidase		N/A
pip	Q6MHR0	Proline iminopeptidase		N/A
Bd1518	Q6MMV5	Aminopeptidase	78	Unknown

Table 2. Proteases and peptidases identified by mass spectrometry within the HIB supernatant. The size range used for the analysis was approximately between 30 and 70kDa (Dwidar, Monnappa et al, 2014)[32]

To confirm hypothesis about osmotic effects on protease, azo dye impregnated collagen, azocoll and proteases activity assay kits from Abcam is used. Azocoll method is to check the amount of protease in HEPES and 300mOsm/kg, and protease assay kit is for confirming protease activity under osmotic pressure. The processes of azocoll and proteases activity assay kits are mentioned above in material and method part. The result is on figure 7.

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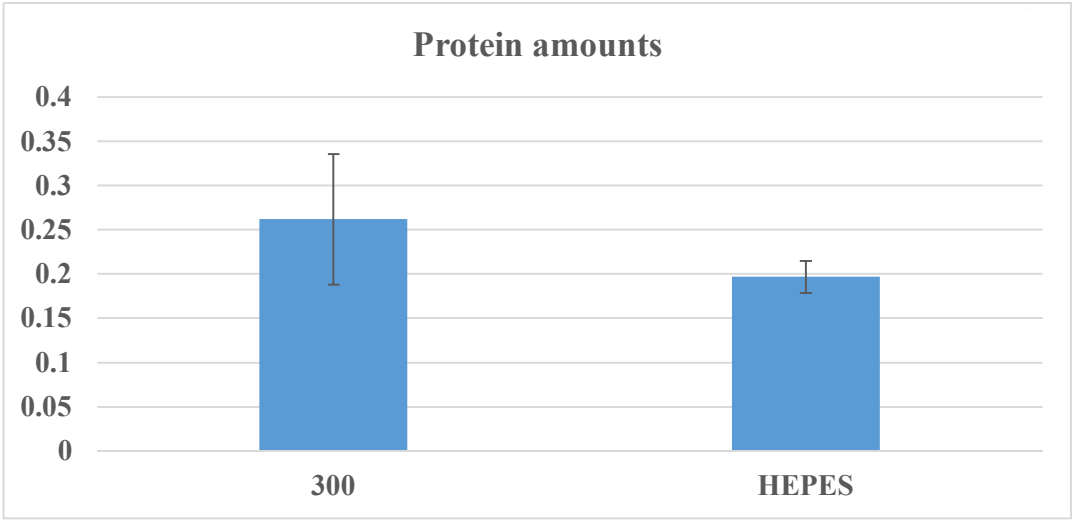
a)



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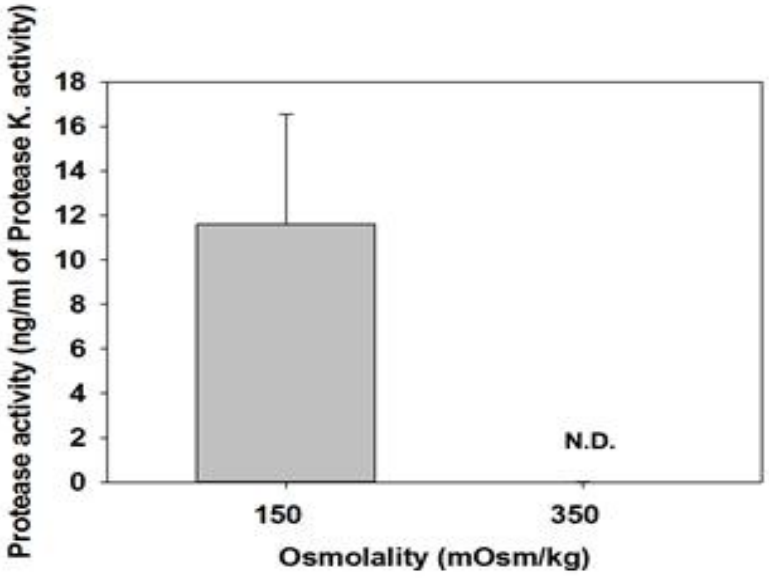

Figure 7. (a) Protease amount assay with azo-dye in 150mOsm/kg and 350mOsm/kg of NaCl solution (n=3). (b) Protease activity assay in 150mOsm/kg and 350mOsm/kg of NaCl solution (n=3)

To get more proteases from BALOs, five times higher concentration of nutrient broth (NB) media was supplied to the *Bdellovibrio bacteriovorus* HD100 after mixing with 250mOsm/kg of NaCl solution at ratio of one-to-one.(5X NB has 150mOsm/kg of osmolality) After 24hours, we checked proteases activity in the broth by Abcam protease activity assay kits.

In figure 7, high osmolality has critical impact on the proteases derived from BALOs. As previous figures, 150mOsm/kg of NaCl solution does not give any effects to the BALOs predation, but more than 200mOsm/kg of NaCl solution gives a negative impact on the BALO predation. In figure 7,

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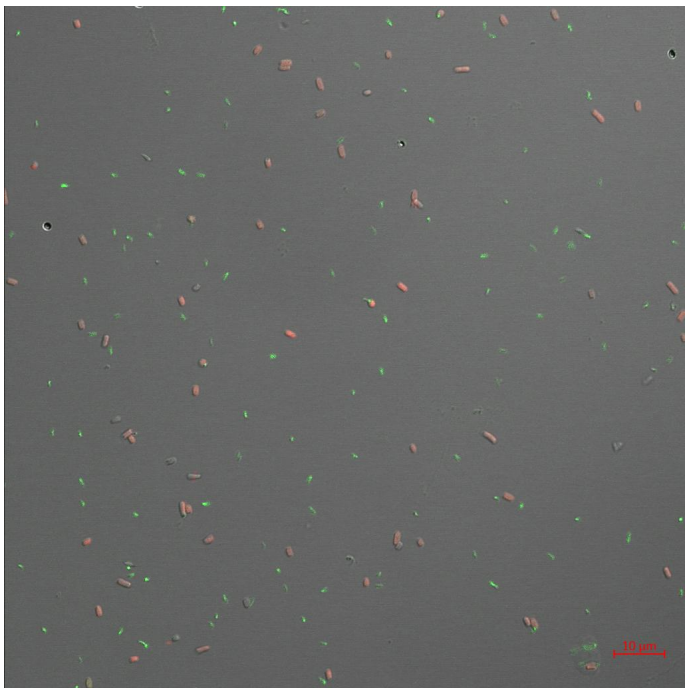
proteases in 350mOsm/kg of NaCl and NB mixture can interrupt their activities. That means proteases have troubles for activating under high enough osmotic pressure.

To confirm the effect on *Bdellovibrio bacteriovorus,* we take the images with confocal microscope. In this step, we use different strain, *E.coli* pHKT3 which produce the Dsred fluorescent proteins and *Bdellovibrio bacteriovorus* HD100 bearing plasmid pMQ572 which produce the Venus yellow protein.[46,47,48,49] The method to prepare the sample is similar to previous case. We mix *E.coli* pHKT3 and *Bdellovibrio bacteriovorus* HD100 with pMQ572 with same ratio and let them grow for an hour at 30℃. After that, the mixture is pelleted, and we resuspend them with same volume of HEPES and 300mOsm/kg. After six hours, we pellet the mixture and take the images. Figure 8 is the result.

a)



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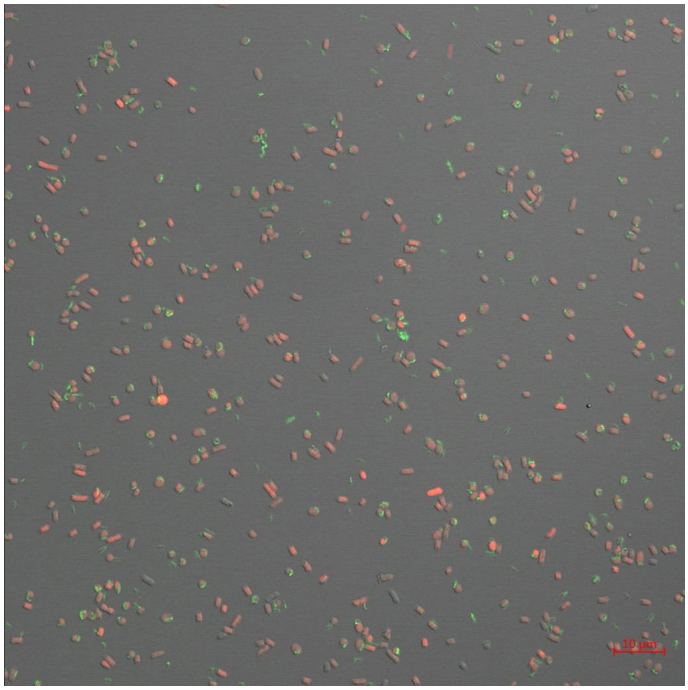
b)



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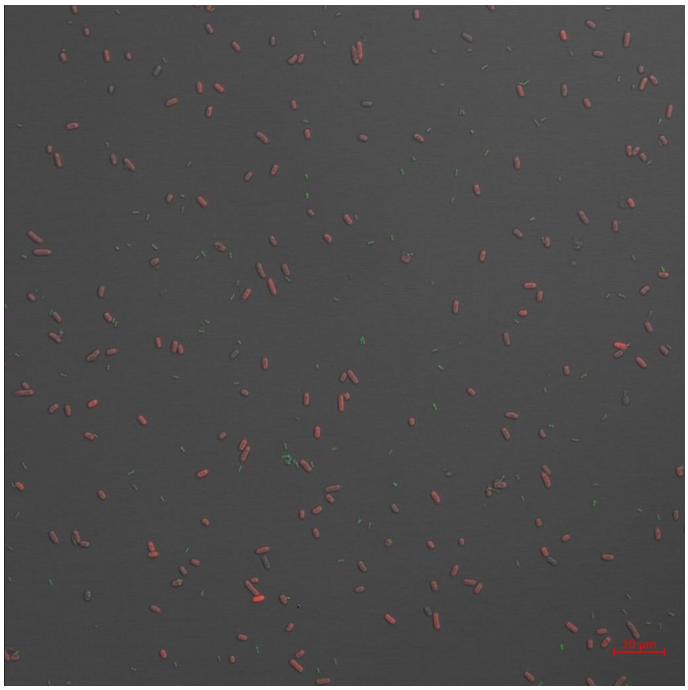

Figure 8. Confocal images of *E.coli* pHKT3 and *Bdellovibrio bacteriovorus* HD100 expressing Venus yellow fluorescent proteins in (a)HEPES(1hr), (b)HEPES(6hr), and (c)300mOsm/kg(6hr).

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According to Figure 8, one hour sample and six hours sample in HEPES show numbers of bdelloplast and plenty of freely swimming *Bdellovibrio bacteriovorus* HD100. However, in 300mOsm/kg six-hour sample, it is hard to find bdelloplast. It can mean that *Bdellovibrio bacteriovorus* HD100 can have trouble to attack the prey under high osmotic pressure.

2. Predation of different *Bdellovibrio bacteriovorus* in NaCl solution against other preys

In part 1, we confirmed osmolality of NaCl solution affects to predation of *Bdellovibrio bacteriovorus* HD100 against *E.coli* MG1655 pucdK and possible hypothesis why such phenomenon occurs. In part 2, we will show the result of *Bdellovibrio bacteriovorus* HD100 predation against other preys like *Klebsiella pneumoniae* and *Acinetobacter baumannii*, and also other BALOs like *Bdellovibrio bacteriovorus* 109J against those preys under osmotic condition.

The protocol for tests with *Klebsiella pneumoniae* and *Acinetobacter baumannii* is the same as *E.coli* case. We mix OD2.0 of preys in HEPES with doubled osmolality of NaCl solution. Figure 9 and Figure 10 are the predation of *Bdellovibrio bacteriovorus* HD100 against *A.baumannii* and *K.pneumoniae*.

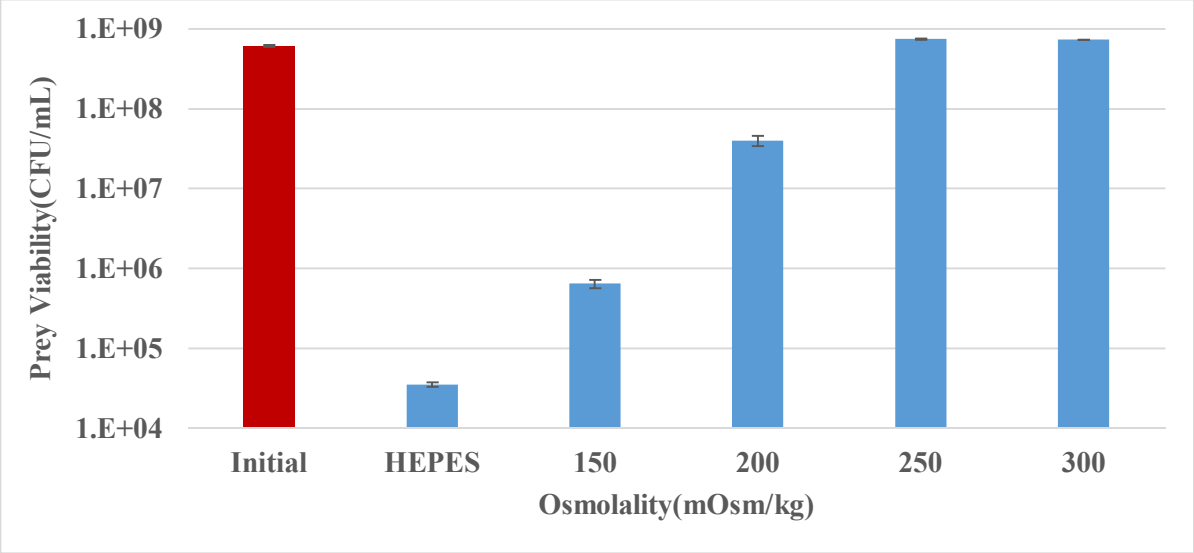

Figure 9. Predation of *Bdellovibrio bacteriovorus* HD100 against *Klebsiella pneumoniae* under different osmolality of NaCl solution (n=3)

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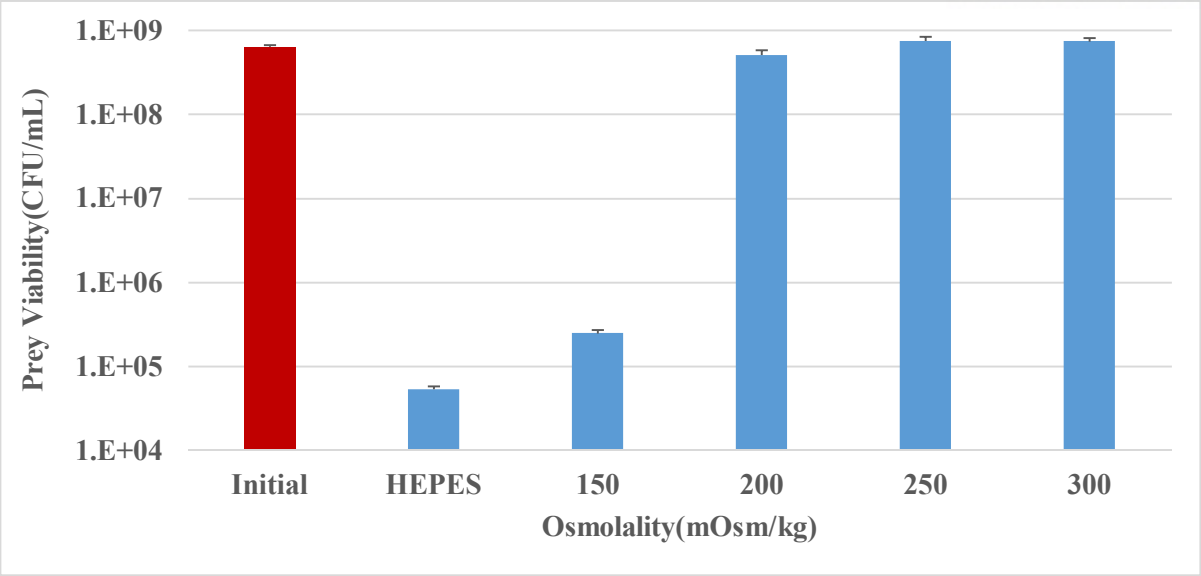

Figure 10. Predation of *Bdellovibrio bacteriovorus* HD100 against *Acinetobacter baumannii* in different osmolality of NaCl solution (n=3)

The *Bdellovibrio bacteriovorus* HD100 used in these experiments was grown with *E.coli*, so it may have problems to predate the new strain, *A.baumannii* and *K.pneumoniae*. Therefore, it can have different predation tendency. Under NaCl solution, *Bdellovibrio bacteriovorus* HD100 can predate *E.coli* in 200mOsm/kg solution, but in the case of *A.baumannii* and *K.pneumoniae*, under 200mOsm/kg of NaCl solution, it is hard to say there is predation. In 150mOsm/kg of NaCl solution, *Bdellovibrio bacteriovorus* HD100 can predating *E.coli* as much as HEPES condition. However, in the case of *Bdellovibrio bacteriovorus* HD100 predate *A.baumannii* and *K.pneumoniae*, it seems to have difference between 150mOsm/kg of NaCl solution condition and HEPES condition, but difference is not huge enough to say *Bdellovibrio bacteriovorus* HD100 face the trouble. Under the higher osmolality, *Bdellovibrio bacteriovorus* HD100 predates preys under, the more preys survive after 24 hours.

There are many other *Bdellovibrio bacteriovorus*. We need to check whether other *Bdellovibrio bacteriovorus* can also be affected by osmolality. *Bdellovibrio bacteriovorus* 109J is one of species of *Bdellovibrio bacteriovorus*[35,36,37]. Additionally, it is *Bdellovibrio bacteriovorus* living in the fresh water. It means there is possibility that osmolality can be a severe factor for *Bdellovibrio bacteriovorus* 109J to predate preys. Therefore, following this, we will show how osmolality affects to *Bdellovibrio bacteriovorus* 109J predation.

The protocol for the test with *Bdellovibrio bacteriovorus* 109J is same as *Bdellovibrio bacteriovorus* HD100 case. They have predated *E.coli* since they inoculated from the stock in -70℃. They have cultured three to four times since they inoculated from the stock as well. Following Figure 11, Figure 12, and Figure 13 are the result of *Bdellovibrio bacteriovorus* 109J against each prey like

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E.coli, A.baumannii, and *K.pneumoniae*.

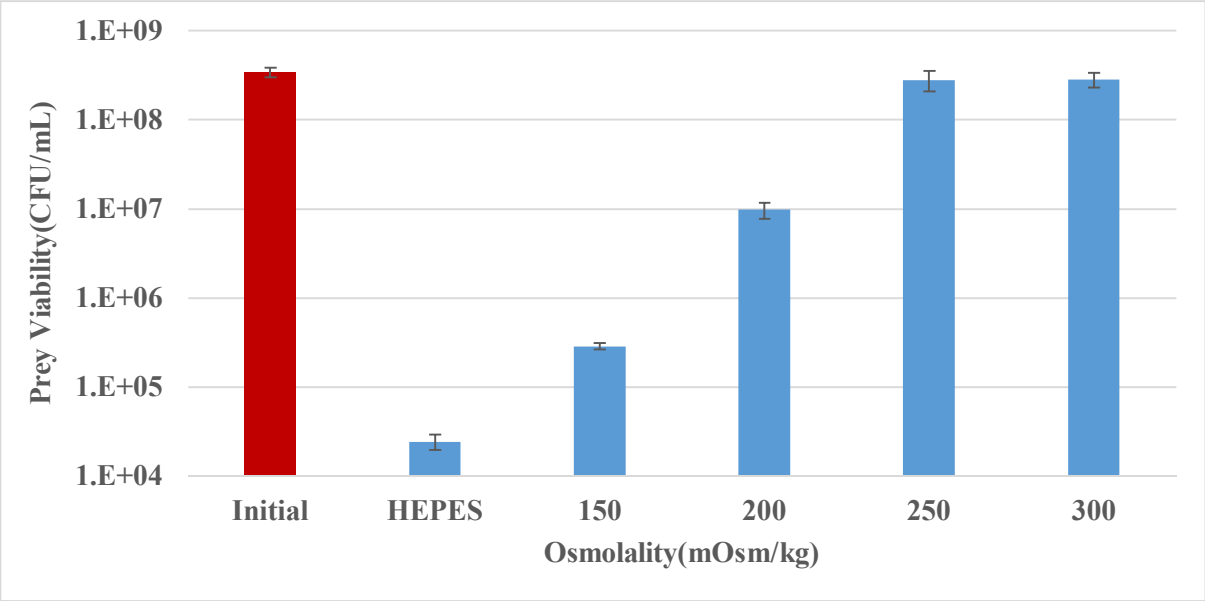

Figure 11. *Bdellovibrio bacteriovorus* 109J predation against *E.coli* MG1655 pucdK under different osmolality of NaCl solution (n=3)

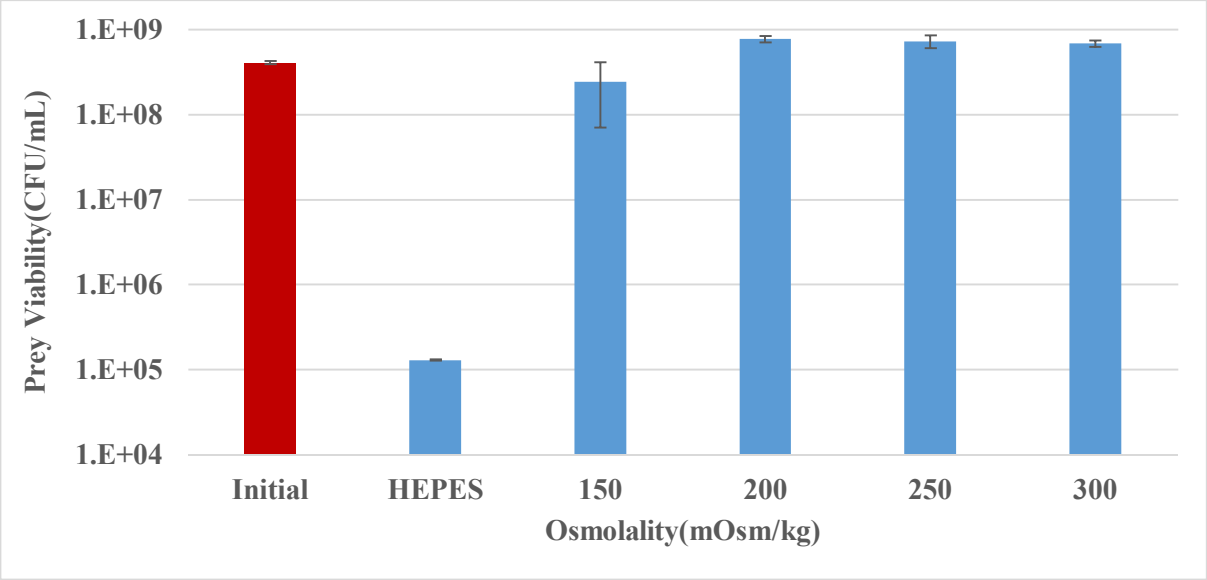

Figure 12. *Bdellovibrio bacteriovorus* 109J predation against *A.baumannii* Clinical Isolates 1 under different osmolality of NaCl solution (n=3)

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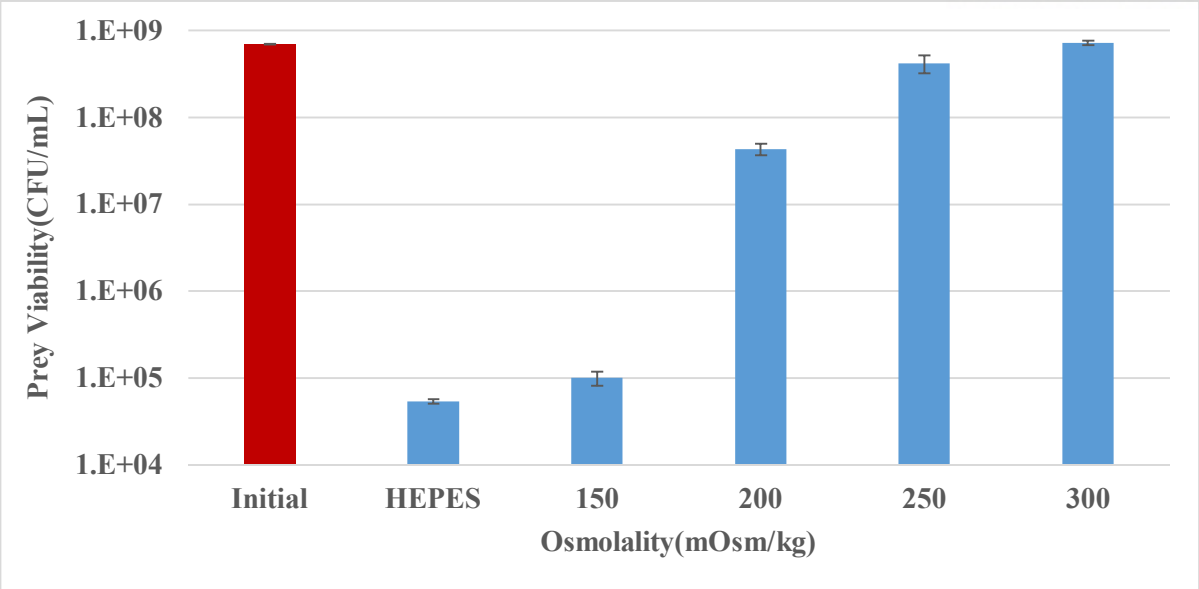

Figure 13. *Bdellovibrio Bacteriovorus* 109J predation against *Klebsiella pneumoniae* under different osmolality of NaCl solution (n=3)

If we compare the result of *Bdellovibrio Bacteriovorus* 109J case to *Bdellovibrio bacteriovorus* HD100 case, it is almost same. However, in the case of *A.baumannii*, *Bdellovibrio bacteriovorus* 109J cannot predate as much as *E.coli*. Despite of it, *Bdellovibrio bacteriovorus* 109J can still predate better under lower osmolality condition because difference between predation rate under HEPES and 150msom/kg of NaCl condition is similar to *Bdellovibrio bacteriovorus* HD100.

To confirm how much osmolality is critical to *Bdellovibrio bacteriovorus* 109J, we confirm the bdelloplast viability in six hours. The protocol of the test is same as *Bdellovibrio bacteriovorus* HD100 case. The number of *Bdellovibrio bacteriovorus* 109J after 24hour-predation is about 1.8E+08 per milliliter. Therefore, triple concentrated *E.coli* MG1655 and double concentrated *Bdellovibrio bacteriovorus* 109J have almost similar number of cells. The following Figure 16 is the result of bdelloplast growth of *Bdellovibrio bacteriovorus* 109J against *E.coli*.

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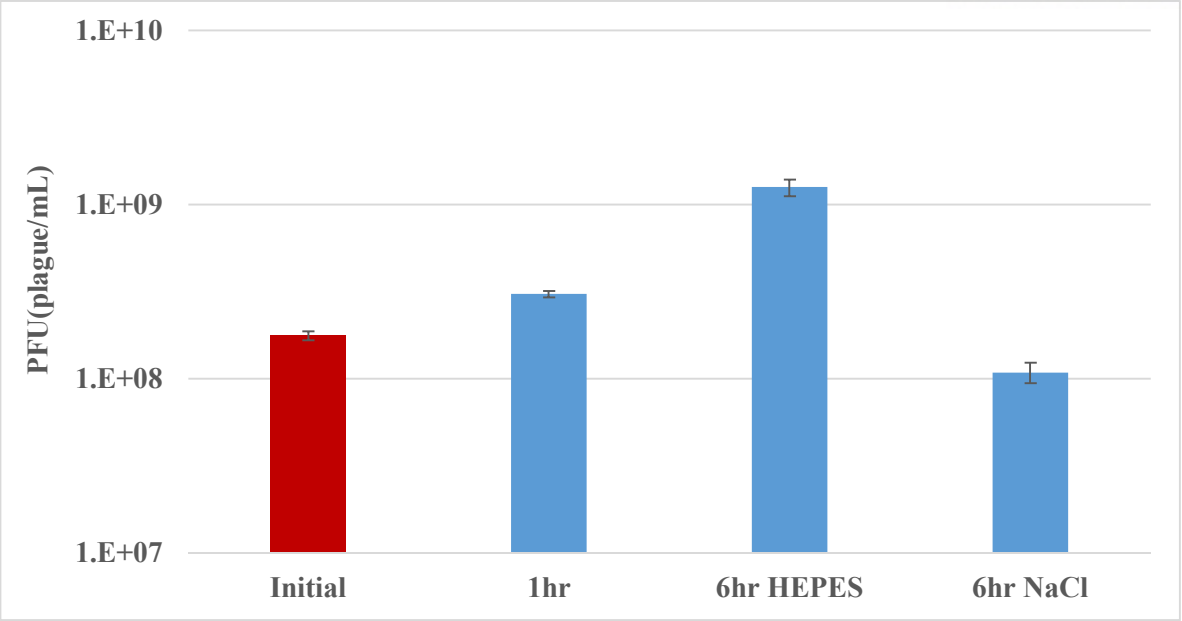

Figure 14. Bdelloplast growth test of *Bdellovibrio bacteriovorus* 109J under 350mOsm/kg NaCl solution (n=3)

If we compare Figure 14 to Figure 6, both have similar tendency like bdelloplast viability decrease, so we can say bdelloplast of *Bdellovibrio bacteriovorus* 109J face the problem because of the osmotic pressure. By comparison of 0hr sample with 6hr NaCl sample and 6hr HEPES each, in HEPES condition, after six hours, the number of plague increases about five times more, but in NaCl condition, after six hours, the number of plague decrease about 40%. That means, osmolality of NaCl solution gives a critical impact on the bdelloplast of *Bdellovibrio bacteriovorus* 109J same as *Bdellovibrio bacteriovorus* HD100.

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3. Predation of *Bdellovibrio bacteriovorus* under different salt solution

From part1 and part2, osmolality of NaCl solution affects the predation of BALOs regardless of the prey strains. When NaCl dissolves in water, it divides into two ions, Na^+ and Cl^-. Like this, other inorganic molecules dissolve in water, and they are separated into two or more ions. That mean, depending on the sorts of molecule, their osmolality is different from each other. In part3, we will show the effects of different salt solution to the BALO predation.

First of all, to check the difference in osmolality of each molecules, we test the relationship between molar concentration and osmolality in room temperature. Figure 15 is the result of the test.

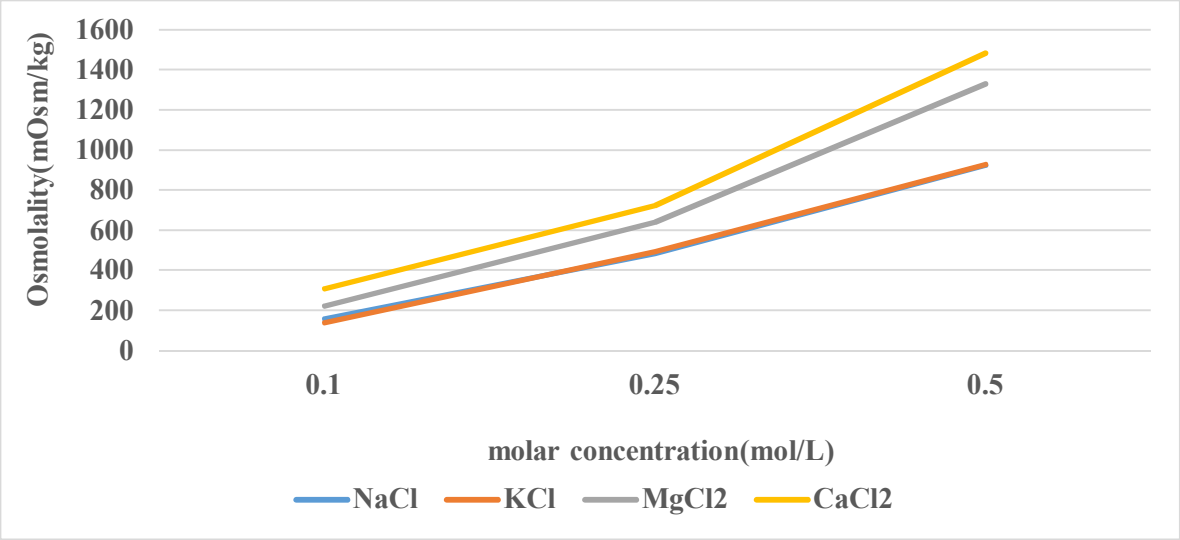

Figure 65. The relationship between molar concentration and osmolality of each salt solution

According to Figure 15, NaCl and KCl have almost similar osmolality to each other, so we can predict NaCl and KCl possibly give similar effects to the BALO predation. That means, the result of KCl test would show similarity to NaCl data shown in previous figure. However, in the case of $MgCl_2$ and $CaCl_2$, both are used for BALO salt which is necessary for *Bdellovibrio bacteriovorus* to mobilize. Therefore, we predict both give less effects on the BALO predation activity. In followings, we will show the result of the test with different salt in multiple ranges of osmolality.

The basic method for the test with different salt solutions is the same as previous NaCl case. To make certain osmolality of each salt solution, the osmometer is used after following the graph of Figure 14. In this test, we used *Bdellovibrio Bacteriovorus* HD100. Each prepared salt solution has the same osmolality to each other. In the test, we used 150msom/kg, 250mOsm/kg, and 350mOsm/kg of each solution and HEPES mixture. The results are on the Figure 16, Figure 17, and Figure 18.

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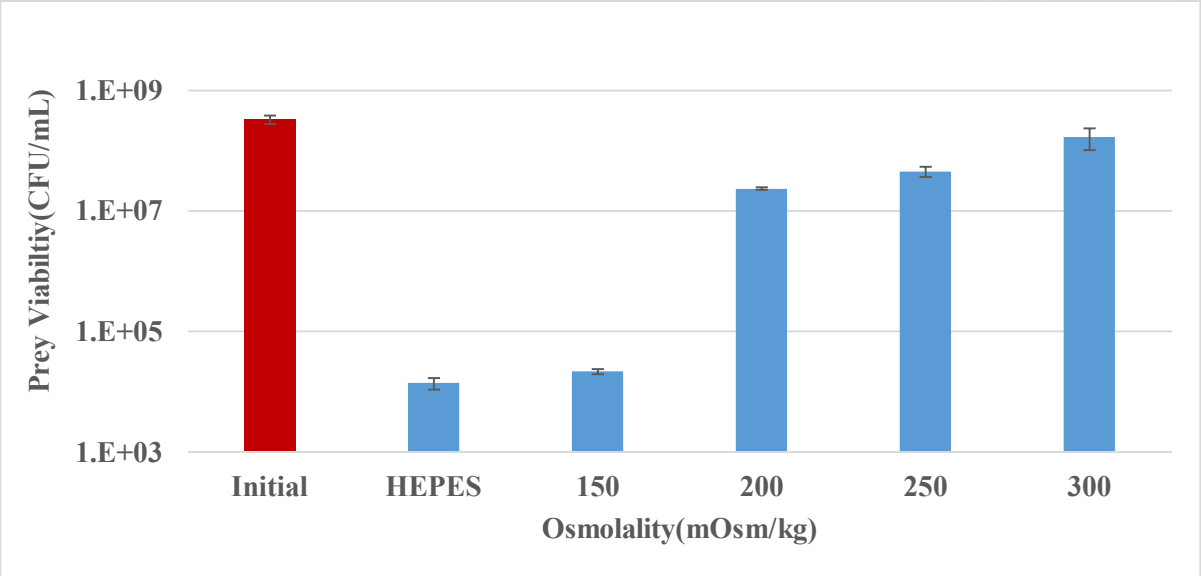

Figure 76. Predation activity of *Bdellovibrio bacteriovorus* HD100 under different osmolality of KCl (n=3)

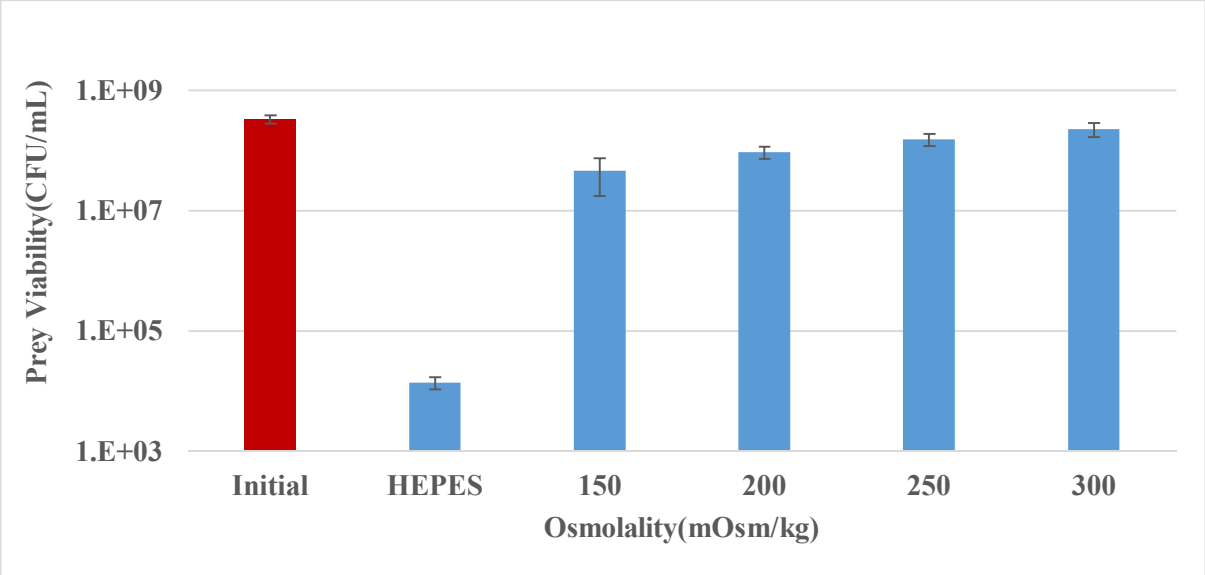

Figure 17. Predation activity of *Bdellovibrio bacteriovorus* HD100 under different osmolality of $MgCl_2$ (n=3)

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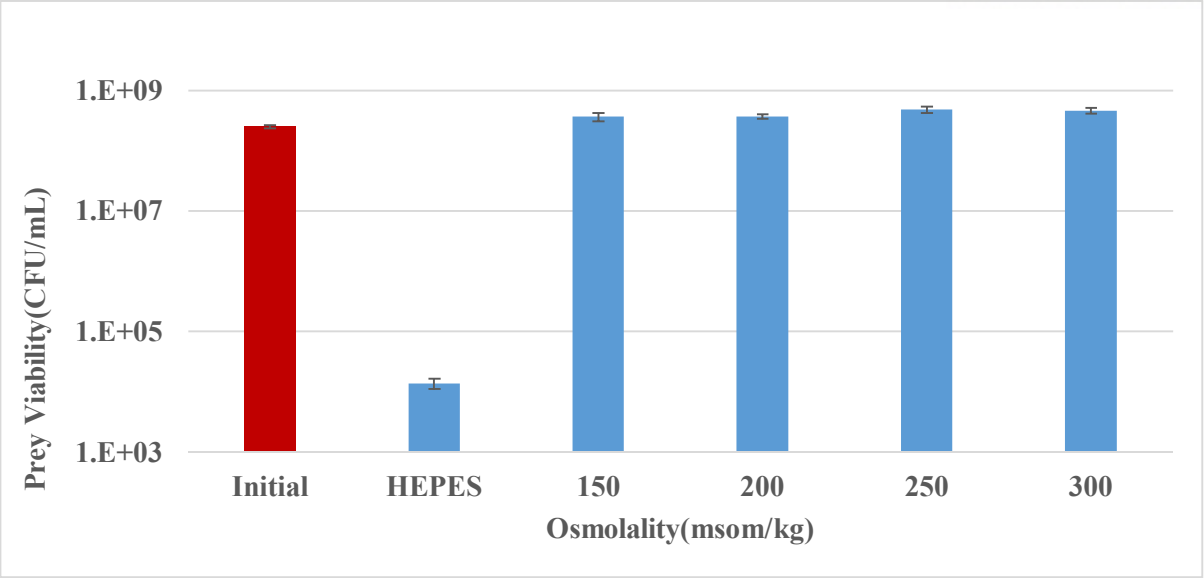

Figure 18. Predation activity of *Bdellovibrio bacteriovorus* HD100 under different osmolality of $CaCl_2$ (n=3)

According to figures above, we can notice that each salt solution gives different effects to BALO predation despite of same osmolality. If you compare NaCl data (Figure 4.) and KCl data (Figure15), we can notice they look similar, but there is difference in the case of 200mOsm/kg condition. Under 200mOsm/kg of NaCl solution, there is predation, but in the case of KCl, there is no predation. In the case of $MgCl_2$ and $CagCl_2$, there are difference under predatable range of NaCl, from 150mOsm/kg to 250mOsm/kg. Under 150mOsm/kg case, in the case of $MgCl_2$, there is predation according to the figures, but there is not any predation under 150mOsm/kg of $CaCl_2$ in comparison of prey viability after 24hours. It implies that $CaCl_2$ is the most impactful to the BALOs' predation activities compared to NaCl, KCl, and $MgCl_2$.

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4. Prey stability under osmolality condition

At this point, we wonder whether osmolality does not have any negative impacts to the prey. Therefore, we do the test with prey viability under osmotic conditions. This test is done with the basic method of predation test, but we do not add any *Bdellovibrio bacteriovorus*. The results are on the Figure 19, Figure 20, and Figure 21.

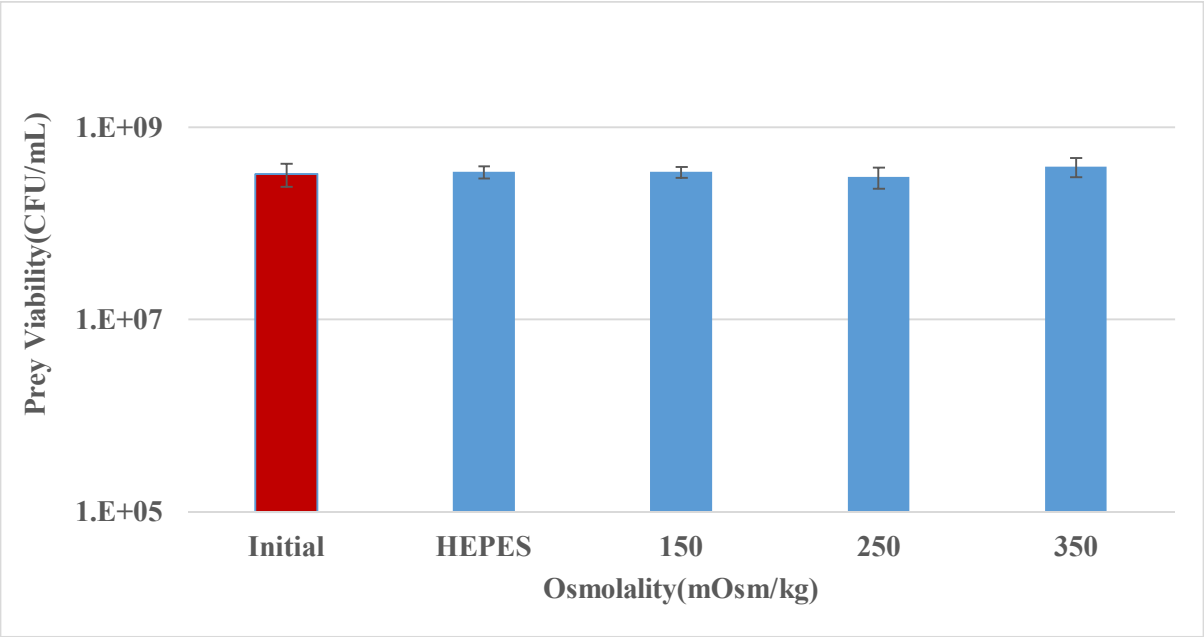

Figure 19. *E.coli* MG1655 pucdK viability under different osmolality of NaCl solution (n=3)

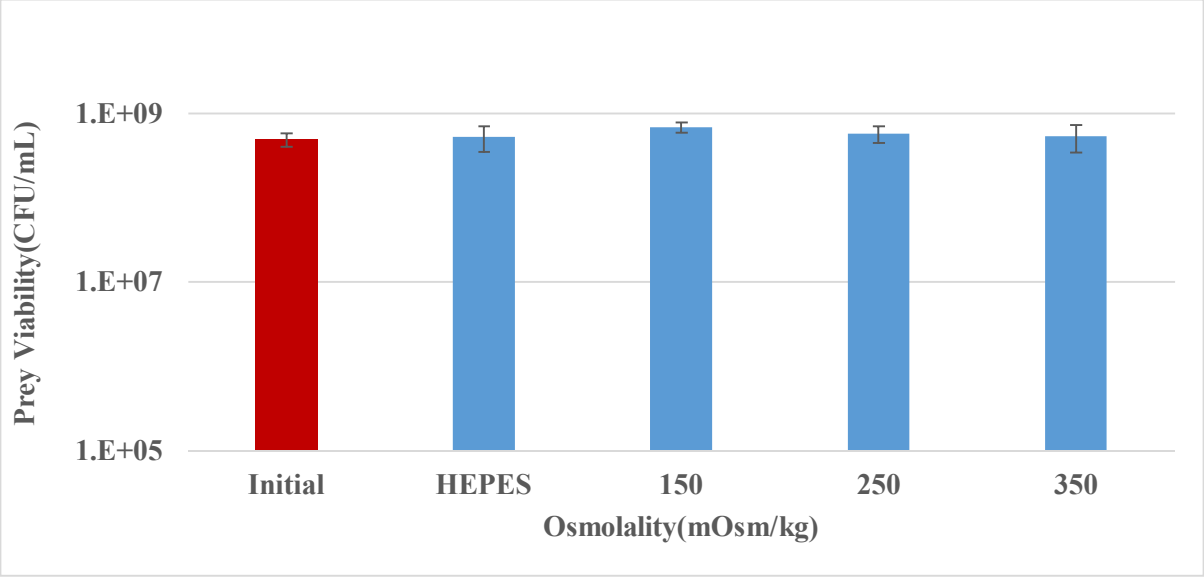

Figure 20. *Klebsiella pneumoniae* viability under different osmolality of NaCl solution (n=3)

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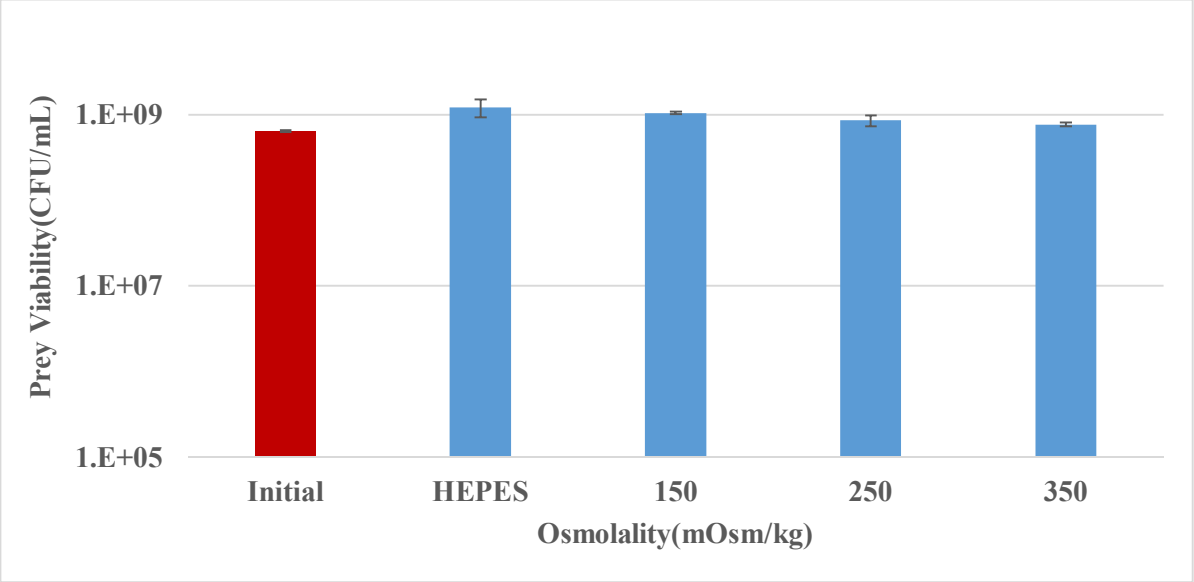

Figure 21. *Acintobactor baumannii* viability under different osmolality of NaCl solution (n=3)

According to the figures, we can notice that high osmolality does not give any effects to the preys themselves because prey viability of initial and 24hr-grown preys in the different osmolality solutions are almost same. Therefore, we can say osmolality gives more negative impacts on the predator bacteria than preys.

5. Predation in organic compound solutions

Previous tests are done with inorganic compounds which are soluble and ionized under aqueous condition. Moreover, ions from inorganic compounds have strong electric charge which can also be a factor for *Bdellovibrio bacteriovorus* predation. In other words, there is possibilities that inorganic compounds cause troubles to the *Bdellovibrio bacteriovorus* predation activities because of their electric charge. To figure out whether the radical reason for osmolality is strong electric charge of ions from inorganic compounds, we do experiments with organic compounds.

Among several possible candidates for organic compounds, we chose sugar and amino acids because they are used in organisms as a nutrients. Moreover, they also can have osmolality in the aqueous solvents according to Figure 21 and Figure 22. In this part, we will show you how sugar and amino acids affect to the BALO predation.

The experiments for confirming the effects of sugar and amino acid are used the same protocols as the test above. However, first of all, we have to choose the exact candidates and check the osmolality

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of sugar and amino acid. In this part, we choose sucrose as the sugar and tryptone as the amino acids. The reason for choice of sucrose is not utilized neither nitrogen nor carbon source by *E.coli* according to the data of biolog phenotype microarray from ECOCYC, a member of the BIOCYC database collection. For the experiments with Tryptone, we choose different preys, *E.coli* ArcAB.[38]

The first thing to do is checking osmolality and molar concentration or percentage concentration relationship at room temperature because the molar weight of tryptone is not noticed from the company, BECTON. Figure 22 and Figure 23 are osmolality-molar/percentage concentration relationship of sucrose and tryptone.

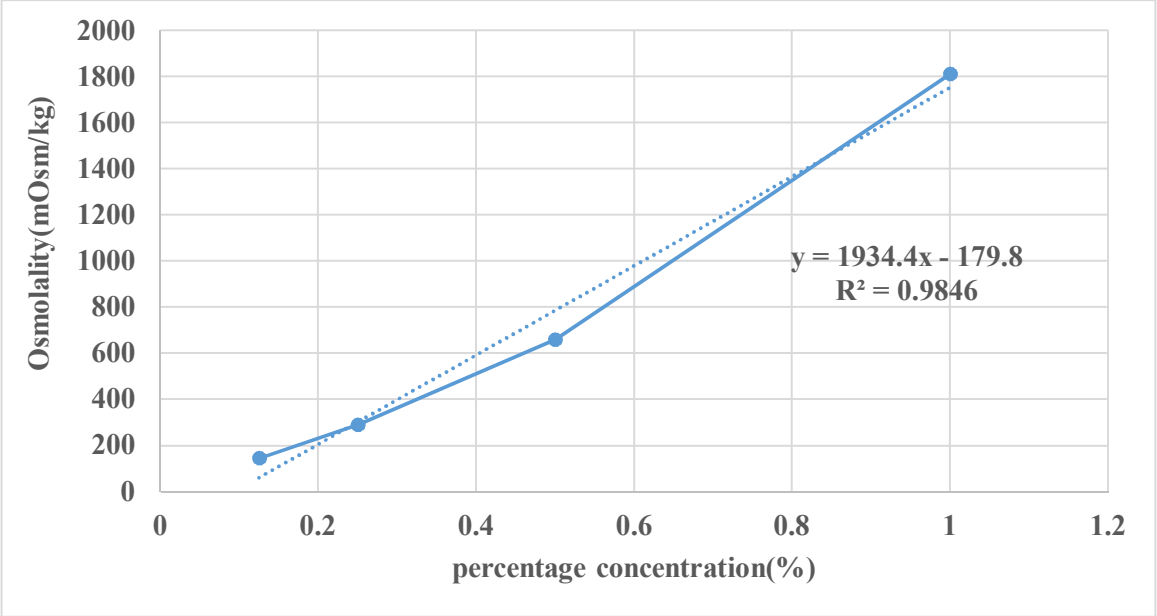

Figure 22. Relationship between percentage concentration and osmolality of tryptone

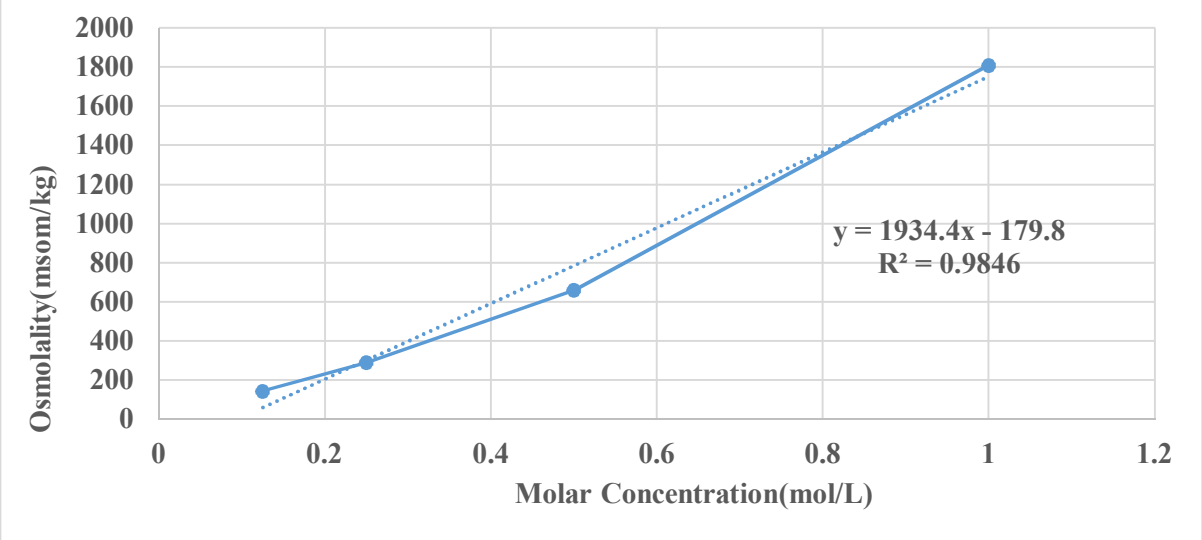

Figure 23. Relationship between osmolality and molar concentration of sucrose

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According to Figure 22 and Figure 23, we can notice osmolality of sucrose is almost similar to $CaCl_2$ and $MgCl_2$. If we compare tryptone to NaCl, we can notice osmolality of tryptone is much higher than NaCl at the same point. 1% of NaCl has about 300mOsm/kg, but 1% of tryptone has about 1800mOsm/kg, about six times higher than NaCl.

The protocol is the same as other experiments and done following the figures above. In these experiments, we change the prey from *E.Coli* MG1655 pucdK to *E.Coli* AcrAB DAP mutant[38]. DAP mutant is mutated in the gene which produces DAP, diaminopimelic acid that is necessary components of peptidoglycan. Therefore, DAP mutant does not grow without diaminopimelic acid. In other words, DAP mutant does not grow under sucrose and tryptone solution. The results of testing the effects of sucrose and tryptone on *E.coli* AcrAB are on the Figure 24 and Figure 25.

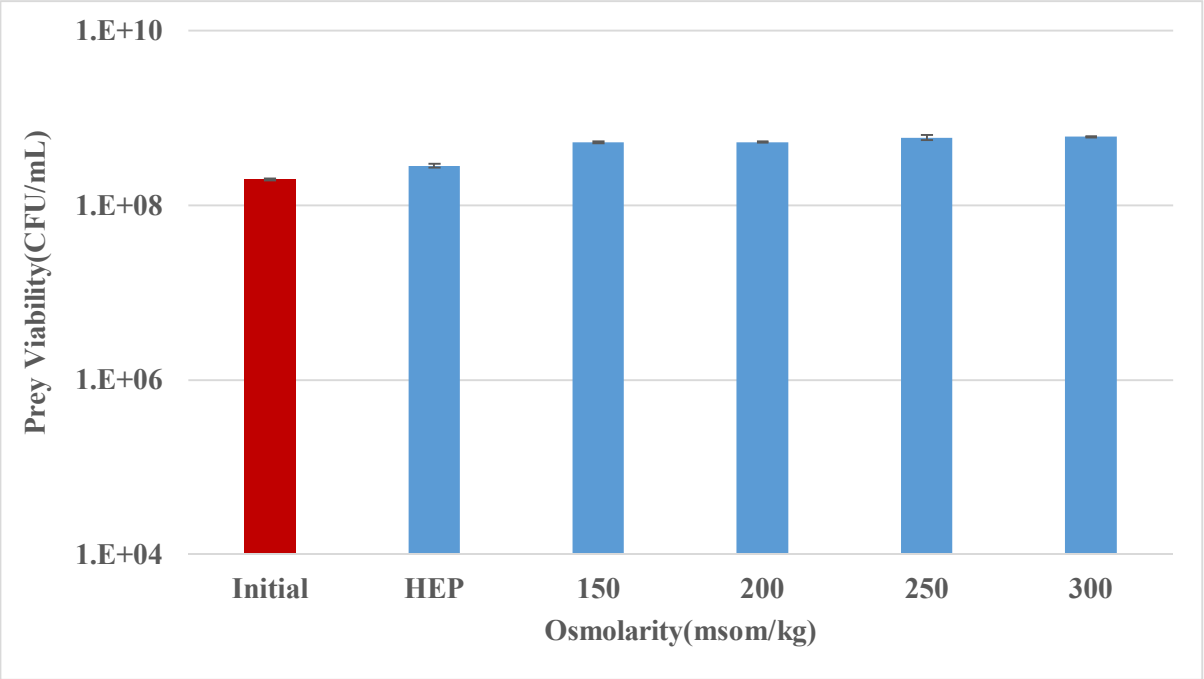

Figure 24. *E.coli* AcrAB viability after 24hours adding tryptone (n=3)

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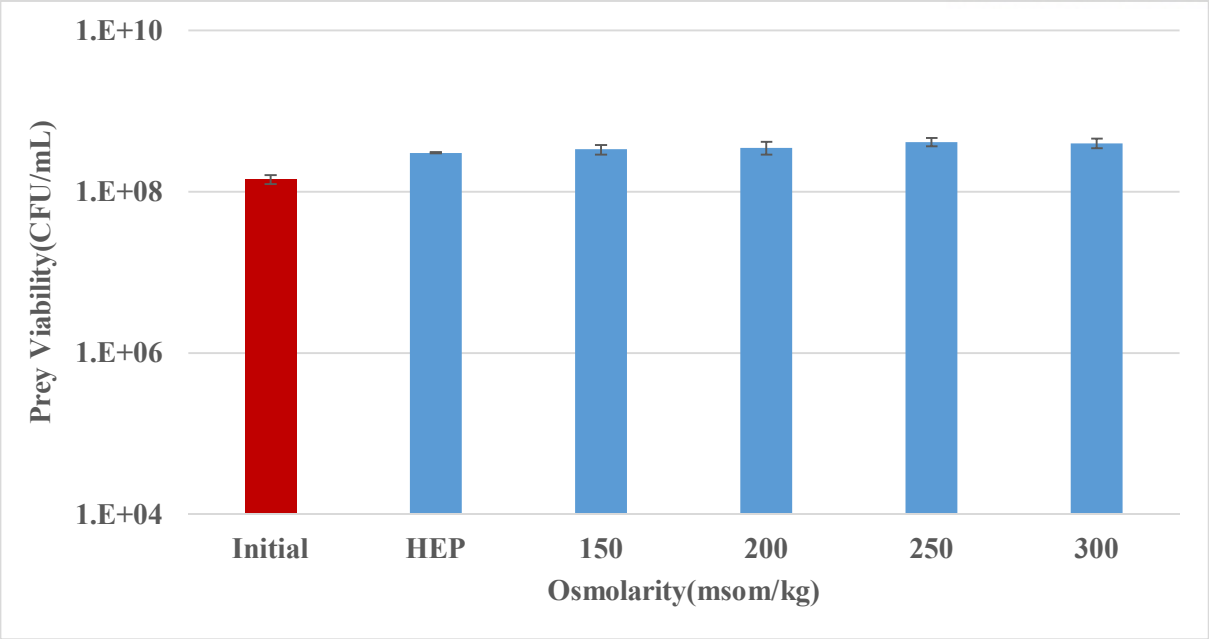

Figure 25. *E.coli* AcrAB viability after 24hours adding sucrose (n=3)

According to Figures, It seems there is little growth in both sucrose and tryptone, but it is not that seriously grown. Therefore, we can say *E.coli* AcrAB DAP minus mutant cannot grow enormously as well as take any negative impacts under sucrose and tryptone condition. From this, we do not have any problems to use *E.coli* AcrAB DAP minus mutant with sucrose and tryptone. With the same protocol as NaCl test, we did predation test of *Bdellovibrio bacteriovorus* HD100 against *E.coli* AcrAB DAP minus mutant. Figure 26 and Figure 27 are the results of predation under sucrose and tryptone.

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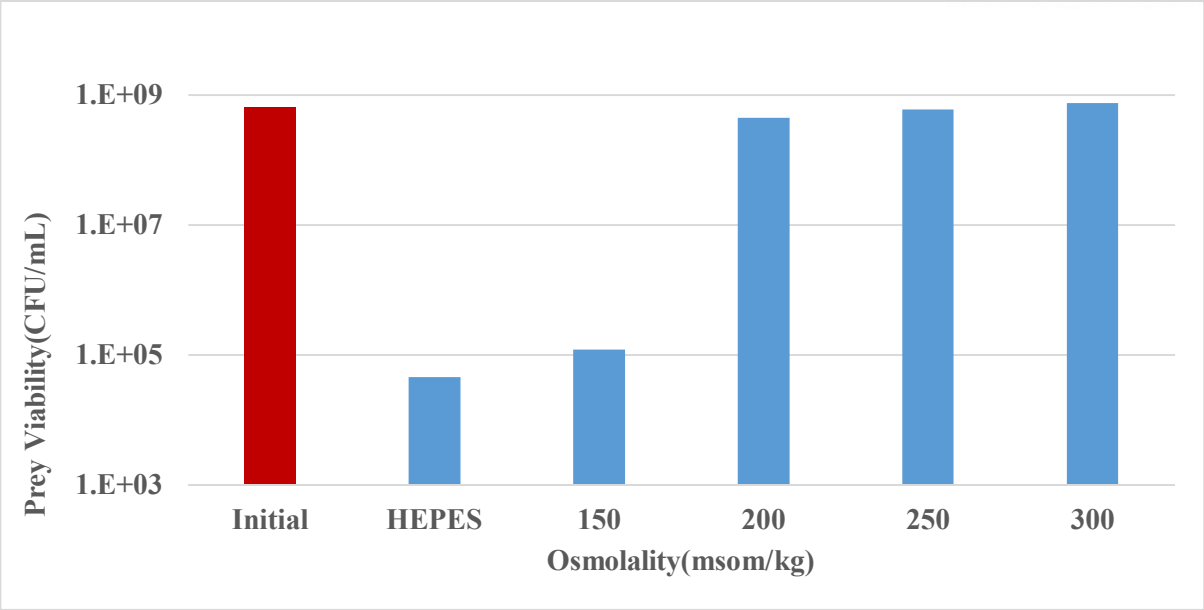

Figure 26. Predation of *Bdellovibrio bacteriovorus* HD100 under different osmolality of tryptone (n=3)

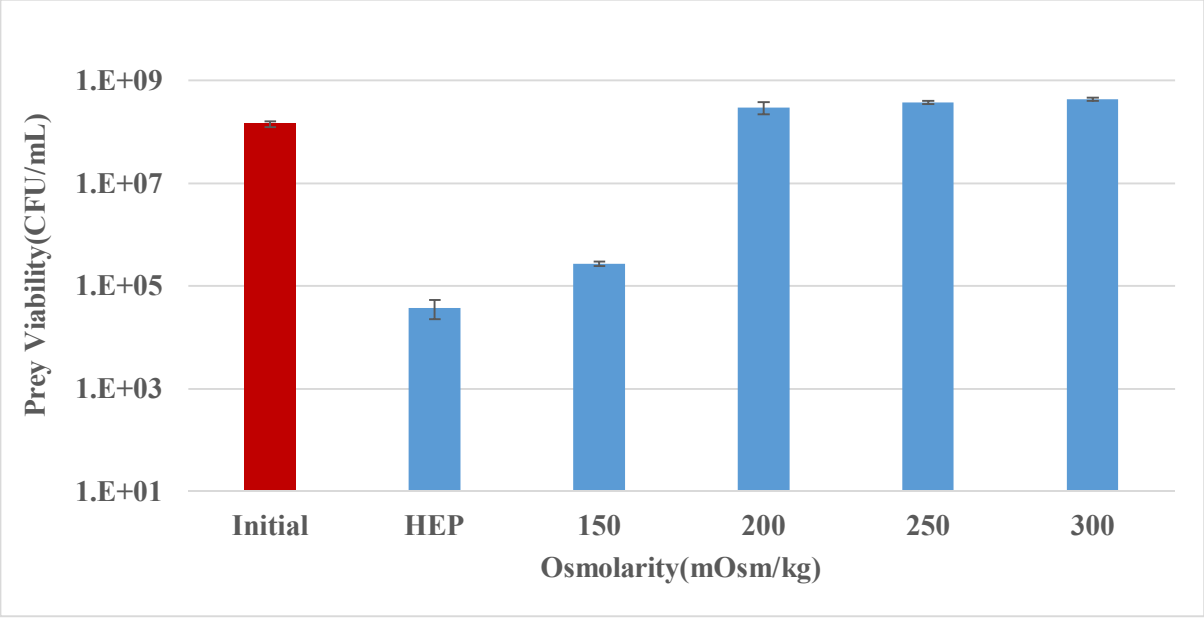

Figure 27. Predation of *Bdellovibrio bacteriovorus* HD100 under different osmolality of sucrose (n=3)

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According to Figure 28 and Figure 29, we can say not only inorganic compounds but also organic compounds solution can affect predation activities of *Bdellovibrio bacteriovorus* HD100. Tendency of both experiments is similar to previous cases. There is predation in 150mOsm/kg, but over 200mOsm/kg of solution prevents *Bdellovibrio bacteriovorus* HD100 from predating preys in the solution. Previous inorganic compounds are consist of cations and anion, Cl^-. However, in this part, we realize organic compounds show similar tendency to inorganic compounds. In other words, we can say *Bdellovibrio bacteriovorus* HD100 is sensitive to the osmolality.

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Conclusion

In this chapter, we show a series of data which are tested with different *Bdellovibrio bacteriovorus*, different preys, and different solutions. Regardless of *Bdellovibrio bacteriovorus* speices, prey species, and solutions, high osmolality like 300mOsm/kg inhibits BALOs from predation, but low osmolality like 150mOsm/kg does not give any obstacles to *Bdellovibrio bacteriovorus*. Therefore, we can conclude that *Bdellovibrio bacteriovorus*, one of the BALOs living in the fresh water is sensitive to the osmolality. At this point, we can say the differences of BALOs from different sites of the river-sea are made by osmolality majorly.

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Chapter 3. Osmolality effects on the real situation

Introduction

1. Summary

From Chapter 2, we can notice osmolality has severe impacts on the BALO predation. In Chapter 3, we will look up the osmolality applied in the real situation. For example, in side of human body, there are multiple different sorts of liquid system like gastrointestinal liquid, lymphatic liquid, and blood. Among these liquid, we will focus on blood because it is hard to find out the live BALOs in the blood. In this part, we will check the effects of osmolality in the real situation.

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The osmolality of blood serum effects on the BALOs

In human body, there are several system using liquid as a main material for circulating and transporting nutrients and other stuff[39,40,41]. For example, gastrointestinal system uses intestinal juice to transport the materials for digestion and nutrients. Lymphatic system use lymph for transportation. Like this, blood is used for blood vessels as a liquid transport material. Among these, we chose blood because it has multiple different aspects possibly influence to the BALOs.

Blood is not simple liquid[42,43]. It is a sort of mixture. Blood is consist of cells and plasma; cells are erythrocytes, leukocytes, and thrombocytes. Plasma contains serum albumin, blood-clotting factors, and immunoglobulins. Such components give high enough osmolality to blood. Also, the ultimate goal of BALO study is application to human body. Therefore, we have to think about the impact of osmolality in blood affecting on the BALOs.

In the case of blood, there is many things to consider to apply at BALO cases. The blood is not simple as much as organic and inorganic solution. In blood, there are multiple different components in, so there are several thing more to consider except for osmolality. For example, in blood, there are many peptides and cells, so it is viscous enough to make a problem because viscous media interrupts BALOs from using flagella to swim around for seeking the prey. Therefore, we have to simplify the experiments.

To simplify the experiments to just confirm the osmolality of the blood components, among them, we made artificial condition which is similar to blood and chose one of several candidates which can affect BALOs predation activities. Among several choice as candidates, serum albumin is considered as the most impactful to BALO predation. The reason for choosing serum albumin as a factor which gives a obstacle to BALOs is that it fights against bacterial strains commonly associated with bacteremic infections. Therefore, we test the osmolality and serum albumin together.

Serum kill the bacteria through making hole on the membrane of bacteria. We have to find the way to inactivate the function of serum. According to previous papers, serum can be inactivated via treatment with 56℃ heat. To figure out the effects on the prey and predator, we confirm the viability after an hour, two hours, and twenty four hours with serum and 56℃ treated serum. First of all, we checked the osmolality of serum. The result on the Figure 1.

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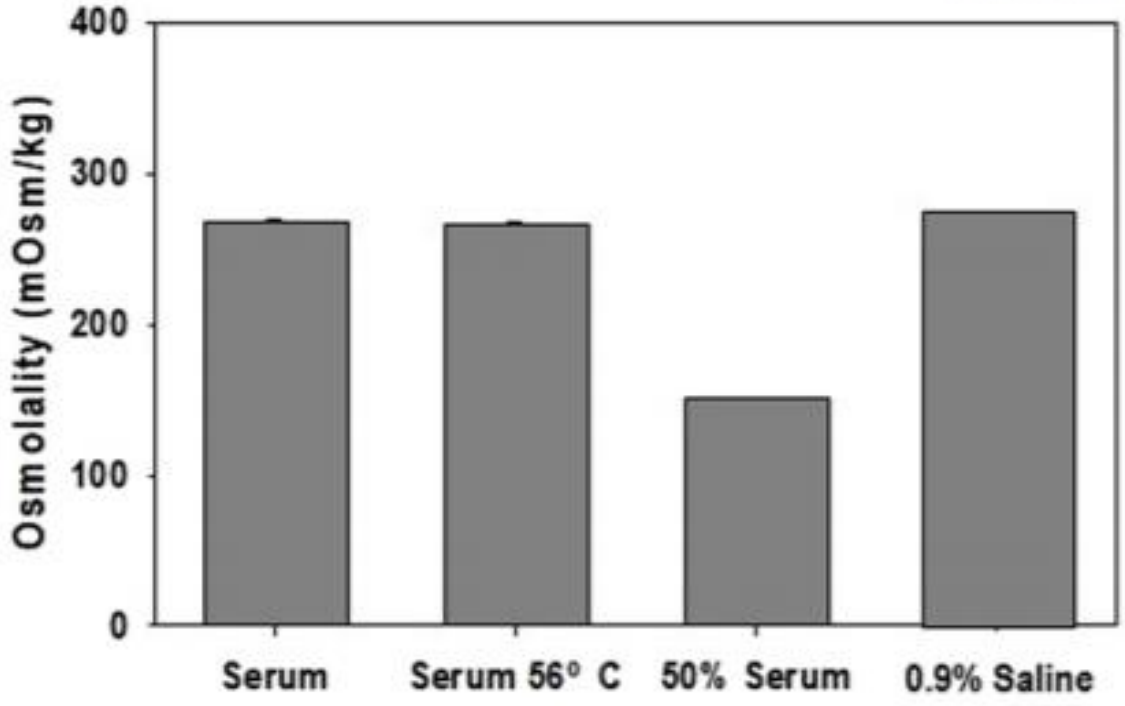

Figure 1. The osmolality of different types of serum

According to Figure 1, heat treatment does not give any changes. The osmolality of serum is 280mOsm/kg, and the osmolality of heat-treated serum is also 280mOsm/kg. Saline has known percentage of NaCl and its osmolality is about 290mOsm/kg. If the result of serum test follows the previous experiments, we can guess heat-treated serum can block the predation activities of *Bdellovibrio Bacteriovorus* HD100.

Serum itself does not kill the BALOs according to figures above. Therefore, we can test the osmolality of serum effect to the BALOs because we can inactivate the serum which kill the prey as well as notice serum itself does not affect to BALO itself. The result of confirmation is on the Figure 5.

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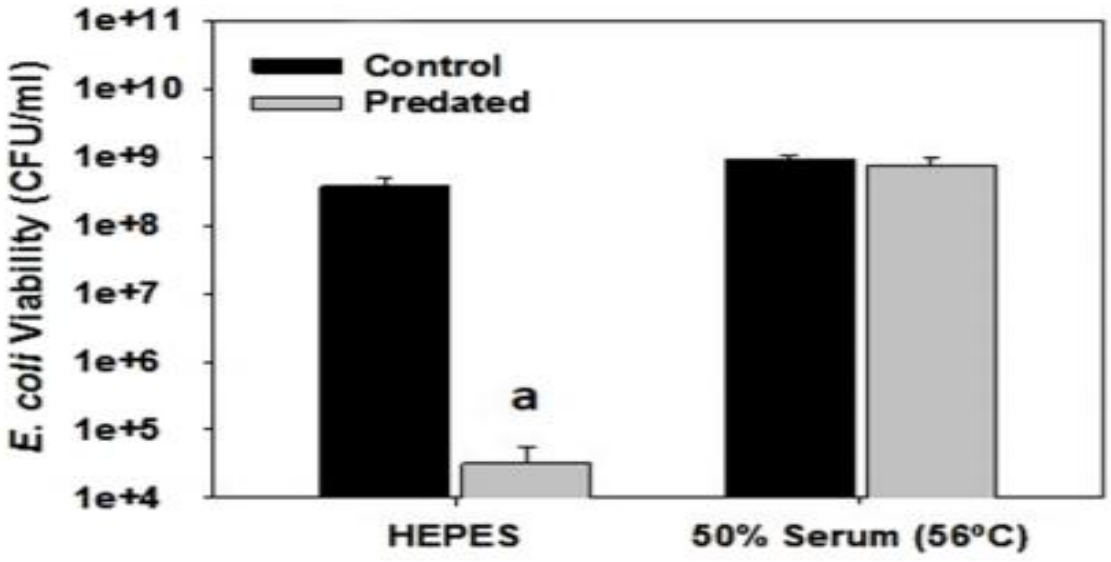

Figure 2. 24hour-predation of *Bdellovibrio Bacteriovorus* HD100 with addition of heat-treated Serum

If you see the Figure 2, we can notice that heat-treated serum which is inactivated and does not give any effects on both BALOs and preys inhibits BALOs' activities. If we consider the osmolality, BALOs should have predated the preys because obstacles from serum albumin are inactivated. However, there is no predation under existence of heat-treated serum. The guess for this phenomenon is that there is another factors for serum albumin which can inhibit the BALOs activities.

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Conclusion

From this Chapter, we can notice the osmolality is able to be applied to the real situation. Human serum albumin is not a single compounds like the materials used in Chapter 2 such as NaCl, KCl, sucrose, and tryptone. Human serum albumin is consist of polypeptides, proteins, so it is hard to say it is ionized by the solvent. However, according to part 2, human serum albumin can induce osmotic pressure, so it has osmolality. Osmolality of human serum albumin in water is about 280mOsm/kg. With such high osmolality, it can interrupt BALOs from predation even though it is inactivated by heat before application to BALOs. That means, osmolality is critical, severe, and impactful to BALOs, but in the enough low range of osmolality for big molecules, other factors can affect as well such as viscosity, pH, and other problematic obstacles.

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Future Work

From Chapter 1 to Chapter 3, we realize BALOs are sensitive to the osmolality. They are much more sensitive than their prey. The conclusion is like that. However, we do not know the exact mechanism how osmolality critically work to the BALOs, and which genes and proteins make different BALOs living in different osmotic conditions. There are several aspects that are not revealed yet. What experiments will have to be followed and which things should be revealed with the following experiments.

First of all, we have to find the basic mechanical difference between BALOs living in the fresh water and marine BALOs. To tract it, we have to isolate the marine BALOs and fresh water BALOs from the same river. Now we are tracking and isolating the BALOs from Taehwa River, Ulsan, Korea. After isolation, it is better to compare genetic difference. We guess there are multiple genes which make marine BALOs resist to the osmolality. That is the point we have to find.

Secondly, we would better check if there is mutation derived from continuous stimulation of osmolality to the BALOs. It is for confirming the correlation between fresh water BALOs and marine BALOs. The adaptability of BALOs to the osmolality will be confirmed. The processes like this: we will keep growing BALOs into osmotic conditions of NaCl. Firstly, we will start to grow BALOs into 150mOsm/kg of NaCl solution which is the range of osmolality that BALOs can act normally. Then, we will use two possible scenario for adaption into the osmolality. One is to keep growing BALOs into 150mOsm/kg of NaCl and transfer to high osmolality. If there is predation in 300mOsm/kg, then we will use it to be adapted higher osmolality, or it will keep in 150mOsm/kg. In other words, we will grow BALOs in 150mOsm/kg for every day, and after 24 hours, we will supply them 300mOsm/kg of NaCl condition for 10days. Then, we will tract the change. The other one is gradually increasing osmolality of BALOs growth condition. Start point is 150mOsm/kg, and the osmolality is gradually increasing 10mOsm/kg per day. We will tract the changes to BALOs.

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